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The Role of Endocrine Factors in the Alteration of Cytochromes P450 by Cyclosporine

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The Role of Endocrine Factors in the Alteration of Cytochromes P450 by Cyclosporine

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To my husband Phil,

whose support and understanding (like his passion for golf)

are never ending

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The Role of Endocrine Factors in the Alteration of Cytochromes P450 by Cyclosporine

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Cyclosporine is an immunosuppressant whose side effects include suppressing cytochrome P450 (P450) levels and activity in the liver. This effect may be one of the underlying causes in the difficulties in relating cyclosporine blood levels with efficacy and toxicity. Growth hormone (GH) is known to pretranslationally regulate P450 expression, while prolactin can interact directly with cyclosporine as an immunomodulator. Thus, the suppression of P450 by cyclosporine may involve GH or prolactin as intermediates. In order to address these hypotheses, the effect of exogenous GH and prolactin, separately and in combination with cyclosporine, on P450 3A1/2 (CYP3A1/2) and 2C11 (CYP2C11) was investigated. In addition, the direct effects of cyclosporine on GH-related cellular signaling factors were examined *in vitro*.

Results from *in vivo* studies revealed that cyclosporine does not alter GH levels as a mediating event in suppressing P450s, although GH is a dominating factor over cyclosporine in determining hepatic P450 expression. The additive suppression in P450 activity seen with the concomitant administration of cyclosporine and GH suggests that changes in hormonal status is likely to be one of many factors that is responsible for the lack of a clear association between cyclosporine dose and toxicity. In the context of prolactin as a mediating factor in the suppression of P450 by cyclosporine, bromocriptine caused a significant suppression of CYP3A1/2 and CYP2C11 protein and activity levels when it was administered alone and in combination with cyclosporine. While cyclosporine and bromocriptine, separately, can significantly alter the fate of hepatic P450 enzymes, the suppression is likely not due to an alteration in prolactin levels.

In vitro studies indicate that P450s are differentially modulated by cyclosporine. While CYP3A1/2 is decreased at specific concentrations, CYP2C11 is increased in a dose-dependent manner. This increase in CYP2C11 correlated with increases in Stat5b binding activity, which initiates transcription of P450 genes in the nucleus.

Taken together, these studies indicate that while cyclosporine does not suppress P450 enzymes by altering GH or prolactin levels, one of the avenues of suppression is through modifying intracellular signaling elements.

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CHAPTER ONE

Introduction and Literature Review

Organ and bone marrow transplantations are widespread procedures that are now carried out with relative success due to the discovery of and treatment with powerful immunosuppressants such as cyclosporine (CsA). The ideal situation for organ transplant immunosuppression would be to prevent immunologic rejection of the donor organ without introducing drug toxicity to the patient. Although CsA produced promising results in the clinical setting, its usefulness was offset by its side effects. One of the most limiting side effects is the onset of organ toxicity. This difficulty in assessing an accurate therapeutic window may be attributed to the existence of large interpatient inconsistencies in pharmacokinetic profiles (Clardy et al., 1988). This problem is further compounded by the fact that there remains a lack of a direct correlation between blood levels of the drug and the extent of pharmacological effect and organ toxicity.

Hepatic cytochrome P450 (P450) enzymes facilitate a large majority of the xenobiotic metabolism that takes place in the liver. Numerous factors can

alter the fate of P450 expression and function, including hormonal status. Much is now known about the mediation of drug metabolism by hormones and the importance of the endocrine system in the regulation of P450 enzyme levels. Altering levels of P450 protein expression can ultimately lead to modified drug metabolism, which in turn, can affect drug efficacy.

CsA is mainly metabolized by the P450 enzyme system. The following proposed studies aim to investigate potential effects of CsA on intracellular signaling mechanisms that are involved in growth hormone (GH) signaling. Additional studies also intend to investigate the role of prolactin as an alternate to GH as a mediating endocrine factor. Results from these studies will provide useful information regarding the regulation of drug metabolism and further clarify the interrelationship between the endocrine system and xenobiotic metabolism.

1.1 CYCLOSPORINE

1.1.1 Background

CsA was first discovered to have immunosuppressive effects by Jean-François Borel in 1972 in fungal cultures of *Typocladium inflatum Gams* (Kahan, 1987). Following extensive testing and upon discovering CsA's selectivity for T-lymphocytes, Sandoz Pharmaceuticals, Corp. patented the first

commercially available form of CsA, Sandimmune® in 1983. CsA is a neutral, highly lipophilic cyclic undecapeptide and is now used as a potent and specific inhibitor of T-cell proliferation. It is composed of 11 amino acids and has a molecular weight of 1202 Daltons (Figure 1.1.1).

1.1.2 Mechanism of action

During the initial phases of CsA's discovery and testing period, it was believed that CsA's inhibition of cell-mediated immunity was achieved through accessory cells (Palay et al., 1986). However, when CsA was administered to cell cultures containing monocytes and dendritic cells, production of IL-2 was not affected, nor was there an observed reduction in monocytes' accessory function for anti-CD3 and lectin responses (Granelli-Piperno et al., 1988). Conversely, when CsA was added to cultures of T lymphocytes, IL-2 production was decreased by 70% and the lymphocytes themselves did not proliferate or release growth factor. These results present direct evidence that CsA primarily blocks the function of T-lymphocytes to achieve immune suppression. In addition, when evaluating the responsiveness of T-lymphocytes to IL-2 following treatment with CsA, it was found that CsA was inhibiting proliferation of T-cells at the level of IL-2 production rather than IL-2 responsiveness.

It was later discovered that when CsA enters the body, it begins its immunosuppressive action by entering T-cells and binding to cyclophilins. Cyclophilins are cytosolic proteins that possess peptidyl-proline-*cis-trans* isomerase (PPIase) activity, which is implicated in protein folding (Schmid, 1995). This CsA-cyclophilin complex now binds and inhibits the Ca^{2+}-activated serine/threonine phosphatase calcineurin. Only the CsA-cyclophilin complex can bind calcineurin; cyclophilin alone does not have this function (Liu et al., 1991). This binding is a crucial step as it prevents calcineurin from binding to cytoplasmic calcium and dephosphorylating nuclear factor of activated T-cells (NF-AT) (Shaw et al., 1995). Normally, following dephosphorylation by the calcineurin- Ca^{2+} complex, NF-AT localizes into the nucleus and forms a complex with AP-1 (Flanagan et al., 1991). This NF-AT:AP-1 complex induces transcription of genes required for T-cell activation, including the IL-2 gene. However, in the presence of CsA, the pathway ends when the CsA-cyclophilin complex binds to calcineurin.

In addition to blocking the NF-AT pathway, evidence has gathered supporting the theory that CsA also affects the activities of nuclear factor-KappaB (NF- κB) and activator protein-1 (AP-1) (Andres et al., 2002; Rincon and Flavell, 1994). The effects on AP-1 are likely mediated by c-Jun NH2-terminal kinase (JNK) and p38. Although the precise mechanism is not known at this time, CsA inhibits JNK and p38 induced activation of T-cells when

stimulated by the T-cell receptor (TCR) and CD28 (Matsuda et al., 1998; Su et al., 1994). It has also been shown that MKK6 and MKK7, two activators of JNK and p38, are both sensitive to the effects of CsA. With this pathway becoming more well characterized, the knowledge of two distinct signaling avenues helps explain the potent and T-cell specific immunosuppressive effects of CsA.

1.1.3 Pharmacokinetic profile

Following an oral dose of CsA, absorption is slow, incomplete, and extremely variable. Peak blood/plasma concentration is reached between one and eight hours after oral administration, but can even occur later. In addition, the absorption half-life ranges between 0.5 and 2.0 hours. The bioavailability between individuals is also highly inconsistent. The observed range is between 2% and 89%, with the average being close to 30% (Lindberg et al., 1986). This large interindividual variability makes accurate dosing difficult. This complexity is also compounded by other interconnected factors such as age, disease status, concomitant drug regimens and variable lipoprotein concentrations (Akhlaghi and Trull, 2002).

Due to CsA's highly lipophilic nature, it distributes widely throughout the body. The highest concentrations are found in the liver and fat, followed by leukocyte-rich organs. Within the blood, CsA preferentially distributes to

various components. In humans, at a concentration of 500 μg/L at 20° C, 58% of CsA is bound to erythrocytes, 33% in plasma (mostly plasma lipoproteins), 4% to granulocytes, and 5% to lymphocytes (Lemaire and Tillement, 1982). In rats following 21 days of CsA administration at 10 mg/kg, the concentration of CsA and its metabolites are found according to the following pattern: skin > fat > liver > muscle > kidney > blood > pancreas (Wagner et al., 1987).

While over 30 metabolites have been identified in human blood, bile, and urine combined, the three main metabolites found in human blood and urine are AM1, AM4N, and AM9 (Kelly and Kahan, 2002). Reactions involved in the metabolism of CsA include hydroxylation, N-demethylation, cyclization, and oxidation (Maurer et al., 1984; Wallemacq et al., 1989). AM1 and AM9 undergo hydroxylation whereas AM4N is an N-demethylated product (Figure 1.1.1). Most of the metabolites are inactive, however AM1 has about 10% of the activity of CsA (Copeland et al., 1990).

The liver, kidney, and intestine are the three main sites of CsA metabolism in humans and rats. CsA is mainly metabolized by the liver where the primary route of excretion is through bile (Maurer et al., 1984). Within the liver, CsA is almost exclusively metabolized by CYP3A (Kronbach et al., 1988; Maurer, 1985) during phase I metabolism, although other isoforms may also be involved (Prueksaritanont et al., 1993b). Therefore, factors that affect the P450 system should also be suspected of affecting the metabolism of CsA. For

example, when hepatic P450 levels were increased in rats, the nephrotoxic effects of CsA were lessened (Cunningham et al., 1985).

During phase II metabolism, only one functional group of the CsA molecule is susceptible to biotransformation. The hydroxyl group of the β-carbon of amino acid 1 can be conjugated. To date, there have been only two phase II metabolites identified in humans: a sulfate conjugate that is found in high concentrations in the bile and plasma of CsA-treated patients (Henricsson, 1990) and a glucuronide that was isolated from bile (Christians et al., 1991).

Elimination of CsA is linear in man, while it is nonlinear in animals. Metabolites are mainly excreted from hepatocytes into bile, although around 3% of a CsA dose is eliminated by the kidney (Bleck et al., 1989). Approximately 90% of a CsA dose is excreted into bile with only 1% of that consisting of parent drug. In addition, less than 1% of a given dose is excreted as unchanged drug into the urine (Beveridge et al., 1981). Approximately 95% of a single oral dose of CsA could be recovered in feces in the form of metabolites over a sampling period of 96 hours (Maurer and Lemaire, 1986). Because metabolites can also have immunosuppressive activity, experimental animals need to be separated from their feces in order to avoid the practice of coprophagia and thus possibly re-dosing themselves with active immunosuppressive metabolites. In humans, the concern lies with the large interindividual variability with respect to clearance. Clearance can differ up to

40-fold in adult renal transplant patients, while the terminal half-life can differ by more than 10-fold.

1.1.4 Toxicity

The chief side effect that limits the use of CsA in the clinical setting is the potential for significant organ toxicity. The main concern lies with nephrotoxicity, however, hepatotoxicity (Kassianides et al., 1990; Welz et al., 1984) and neurotoxicity (de Bruijn et al., 1989; Famiglio et al., 1989) are known to occur as well. In both humans and rats, several toxic effects on the kidney have been documented with the use of CsA.

CsA-induced nephrotoxicity is manifested in two forms: acute and chronic. In the case of acute nephrotoxicity, the abnormality is functional in nature and generally reversible. It is caused by a renal imbalance of vasoconstrictor and vasodilator mediators. More specifically, CsA causes a reduction in renal blood flow (RBF) as a result of constriction of the afferent arteriole as it enters the glomerulus (English et al., 1987). The occurrence of acute nephrotoxicity does not involve pathological changes in the kidney.

Chronic CsA-induced nephrotoxicity is characterized by the presence of pathological damage to the kidney. This pathological impairment is accompanied by functional depression that includes a significant decrease in glomerular filtration rate (GFR) (Caterson et al., 1986). The physical damage is

irreversible and is described as a tubulointerstitial fibrosis in a striped pattern beginning in the medulla and extending into the medullary rays of the cortex (Mihatsch et al., 1995). This pathology is so universal that when searching for suitable animal models of chronic nephropathy, this is the standard to which all successful models are held.

The vasoconstriction associated with CsA use has also been shown to cause occlusion of afferent arterioles, which eventually leads to irreparable glomerular and nephron damage. Although some have attempted to attenuate or reverse the tissue damage altogether, lowering CsA doses in humans has little effect in protecting against microvascular injury seen with high-dose CsA therapy (Myers et al., 1988). CsA-associated arteriolopathy was improved upon discontinuation of CsA therapy following chronic administration to children with steroid-resistant nephritic syndrome. However, other histological damage including tubulointerstitial fibrosis and glomerular lesions did not recover (Hamahira et al., 2001). In rats, a reversal of renal function deterioration, histological damage, and activation of the renin-angiotensin system activity was observed with a 5-week CsA withdrawal period following 5 weeks of dosing (Li et al., 2003). However, the success of CsA withdrawal on reversing nephropathy may be dependent on the dose used and the timing of the drug removal.

During the initial stages of studying CsA-associated nephrotoxicity, research was hampered by the lack of a suitable animal model. The rat proved to be a highly resistant animal to the damaging effects of CsA-induced nephrotoxicity. This may be due, in part, to the high kidney to body weight ratio, which provided for a higher glomerular filtration rate in the rat kidney. While humans displayed renal dysfunction at relatively moderate doses of CsA, an equivalent dose in rats would not produce the same impairment. Thus, it was found that much higher doses of CsA were required to generate comparable dysfunction (Gerkens et al., 1984).

Another obstacle in the development of an appropriate animal model, was the demonstration of the renal tissue damage caused by chronic CsA dosing. However, a solution was found in the sodium-depleted rat model developed by Elzinga's group (Elzinga et al., 1993). Rats were placed on either a sodium-depleted or sodium-replete diet for 14 and 28 days. Following the dosing periods, glomerular filtration rate was not significantly different between the two groups, however, the presence of tubulointerstitial injury was mostly confined to the sodium-depleted group. This demonstrated for the first time an animal model that exhibited the physical damage in the kidney seen in humans following long-term CsA therapy.

In addition to nephrotoxicity produced by CsA, hepatotoxicity is also a significant concern. Both humans and rats experience toxicity to the liver in the

form of hyperbilirubinemia, elevated serum bile acid levels, reductions in bile flow, and reductions in bile acid secretion (Arias, 1993; Kahan, 1987; Roman et al., 1990). These conditions are consistent with CsA being largely eliminated through bile. Along with the apparent consequences on biliary function, CsA also suppresses P450 enzyme expression and activity in the liver of rats (Brunner et al., 1998a). Because CsA is not only an inhibitor of P450s, but also a substrate, the result of this suppression is the potential for a buildup of CsA in the liver that leads to further hepatotoxic effects. In addition to these detrimental effects on hepatic function, it was also found that CsA can cause oxidative stress in rat hepatocytes (Wolf et al., 1997) and increase the growth of carcinogen-induced liver foci (Yabu et al., 1991).

1.2 CYTOCHROMES P450

1.2.1 Background

P450s constitute a superfamily of heme-containing enzymes that are the predominant catalysts of phase I reactions in the biotransformation of a variety of substances (Figure 1.2.1). The chief function of the biotransformation process is to convert lipophilic compounds into more hydrophilic ones in order to facilitate absorption and excretion from the body. The majority of these reactions are oxidations, although they also include reduction and hydrolysis. In

addition, the reactions are not always considered detoxication since P450s also have the potential to convert chemicals to reactive products. The substrates for these enzymes are numerous and broad, including many xenobiotic agents, alcohol, various carcinogens, and even endogenous substances (Vermeulen, 1996). Conversely, many isoforms are selectively inducible by chemical agents. For instance, CYP2E1 is inducible by chronic exposure to ethanol (Ronis et al., 1993) and CYP3A2 is inducible by both dexamethasone (Schuetz and Guzelian, 1984) and phenobarbitol (Wrighton, 1985) in the rat liver.

P450 enzymes are classified into families and subfamilies based on amino acid homology without regard to function. A subfamily is designated with a common letter following the family number. For instance, CYP1A and CYP1B are in the same family, whereas CYP1A1 and CYP1A2 share the same subfamily. In general, a 40% amino acid homology is required for two enzymes to share the same family and 59% is required for two enzymes to belong to the same subfamily (Guengerich, 1997).

1.2.2 CYP3A

The CYP3A subfamily accounts for the majority of drug metabolizing enzymes present in the liver of adult humans and adult male rats (De Waziers et al., 1990). In humans, the CYP3A subfamily accounts for 30% of all hepatic P450s (Shimada et al., 1994) and approximately 70% of all P450s in the

intestine (Kolars et al., 1992). Currently, the isoforms CYP3A3 (Molowa et al., 1986), CYP3A4 (Beaune et al., 1986), CYP3A5 (Aoyama et al., 1989), and CYP3A7 (Komori et al., 1989) have been identified. These isoforms share greater than 80% amino acid homology between each of them. CYP3A4 is the major isoform within the human CYP3A subfamily as it the most highly expressed form in liver and intestine. The list of chemical agents that are metabolized by the CYP3A4 is long and wide-ranging. Some of the xenobiotics include, but are not limited to, cyclosporine, acetaminophen, rapamycin, theophylline, and warfarin (Parkinson, 2001). Even endogenous substances, such as androstanedione, estradiol, cortisol, progesterone, and testosterone (de Wildt et al., 1999) are subject to biotransformation by CYP3A4.

The CYP3A subfamily has clinical importance due to its high expression in the liver and intestine, extensive range of substrates, and its high variability between individuals. As a consequence of its broad substrate specificity, the potential for drug-drug interactions is substantial. The most significant of relationships is the inhibition of CYP3A since this can cause drug toxicity caused by decreased metabolism. One example is the interaction between terfenadine and a known CYP3A inhibitor such as ketoconazole or erythromycin. The reduction in CYP3A-mediated metabolism of terfenadine by a CYP3A inhibitor can lead to such adverse effects as inhibition of cardiac potassium channels, which could cause fatal cardiac arrythmia (Wilkinson,

1996). In addition to xenobiotic effectors, grapefruit juice has also been known to cause potential interactions with drugs that are metabolized by CYP3A. Repeated consumption of grapefruit juice causes a significant increase in CsA AUC levels 24 hours after ingestion (Brunner et al., 1998c). Thus, the consumption of grapefruit juice or any foods containing furanocoumarin compounds should be avoided during therapy with drugs that are substrates for CYP3A. Conversely, inducers of CYP3A can have equally detrimental effects on patients on combination therapy. Rifampin and rifabutin, potent inducers of CYP3A, can increase enzyme expression and activity to the extent that it decreases the concentration of a concomitantly administered drug, thus eliminating its efficacy (Gillum et al., 1993).

The CYP3A4 isoform in humans shares a 72% amino acid homology to the main isoform in male rat liver, CYP3A2 (Maurel, 1996). In the rat, the isoforms CYP3A1, CYP3A2, CYP3A9, CYP3A18, and more recently, CYP3A62 (Gonzalez et al., 1986; Matsubara et al., 2004; Strotkamp et al., 1995; Wang et al., 1996) have been identified. The tissue distribution of these isoforms is broad and includes the liver, small intestine, and brain. CYP3A1 and CYP3A2 are very closely related; they share approximately 89% amino acid homology (Guengerich and Turvy, 1991). For this reason, they share similar substrate specificities and contribute to the catalysis of the same reactions.

Regulation of the expression of the male-dominant CYP3A2 has been attributed to growth hormone (GH) secretion. GH seems to have a repressive role since the abolition of GH by hypophysectomy (removal of the pituitary gland) greatly induced CYP3A2 protein expression and activity as compared to intact male rats (Waxman et al., 1988b). Hypophysectomy also induced the expression of CYP3A2 in female rats even though this isoform is not normally expressed in females at detectable levels. Also, the proper expression of CYP3A2 is dependent on the absence of the female-type continuous secretion of GH. When rats were given monosodium glutamate (MSG) (a chemical known to selectively suppress GH secretion) during neonatal development, intermittent GH pulses administered to male rats restored the expression of CYP3A2 (Waxman et al., 1995a). However, the same pulses administered to female rats rendered GH-deficient by MSG did not induce the expression of CYP3A2 as did with hypophysectomy. This may be attributed to a low basal level of circulating GH still present in females (despite treatment with MSG) that prevented responsiveness to the intermittent administration of GH.

Age is another factor that can alter the expression of CYP3A in humans and rats. Several studies have noted an increase in adverse drug reactions in the elderly population. Postulated causes behind these events include reduced hepatic and intestinal CYP3A, decrease in liver mass, and reduction in hepatic blood flow (Kinirons and Crome, 1997; Schmucker et al., 1990). However, data

on the effects of aging on CYP3A2 in rats has been conflicting. While some data suggest that the level of this enzyme decreases steadily with age (Warrington et al., 2004), others have suggested that there is no significant effect of age on CYP3A (Wauthier et al., 2004). Adding to the controversy, CYP3A in the neonatal rat has been shown to be more responsive to some xenobiotics (Telhada et al., 1992) and less responsive to others (Wright et al., 1997). This indicates that there is a selective drug inducibility on CYP3A expression. It has also been shown that the formation of 6β-hydroxytestosterone, a marker for CYP3A2 activity, is produced at moderate levels in neonates then peaks at adolescence in the male rat (4-12 weeks of age) (Waxman et al., 1985). In adulthood, the activity wanes to a slightly lower level.

One well-established xenobiotic effect on CYP3A is the relationship between CYP3A and CsA. Not only is CsA a substrate for CYP3A but it is also an effector of this subfamily. The suppression of CYP3A2 in rats by CsA is time-dependent and requires a minimum dosing period of 14 days in order to significantly suppress CYP3A2 protein expression and catalytic activity (Brunner et al., 1998a). It was later demonstrated that the dosage of CsA is also a central factor in initiating suppressive action. Moderate daily doses at 15 mg/kg b.w. administered during the requisite 14 days resulted in a significant suppression of both CYP3A2 expression and activity (Brunner et al., 2000).

However, only a dose of 5 mg/kg was required to lower protein expression alone. As a consequence of this inhibitory effect, chronic CsA administration causes increased drug concentrations over time allowing for a higher potential for organ toxicity.

1.2.3 Pregnane X receptor (PXR)

Xenobiotic-mediated activations of P450s are accomplished through the action of various nuclear receptors. Three orphan nuclear receptors, pregnane X receptor (PXR), constitutively active receptor (CAR), and peroxisomal proliferator activated receptor (PPAR) have been identified as mediators of the inductive actions of various exogenous compounds including rifampicin (Bertilsson et al., 1998), phenobarbital (Honkakoski et al., 1998), and clofibric acid (Issemann and Green, 1990), respectively.

Because CYP3A is involved in the metabolism of such a wide range of xenobiotics and endogenous substances and pregnane X receptor (PXR) has been shown to be associated with CYP3A regulation, this makes PXR a pivotal nuclear receptor in the regulation of drug metabolism. There are several lines of evidence suggesting that PXR is the primary determinant for CYP3A gene regulation in mice, rats, rabbits, and humans (Bertilsson et al., 1998; Kliewer et al., 1998; Savas et al., 2000; Zhang et al., 1999). First, PXR is expressed abundantly in the liver and intestine, which are the main organs in which

CYP3A is found (Zhang et al., 1999). Since PXR is present at sufficiently high basal levels, this suggests that not only does PXR play an important role in induction, but also could play a role in constitutive expression of CYP3A as well. Second, PXR forms a heterodimer with 9-*cis* retinoic acid receptor (RXR) and subsequently binds to characterized xenobiotic response elements in human and rat CYP3A promoters. This activates expression of the CYP3A4 promoter in transfection assays (Bertilsson et al., 1998; Lehmann et al., 1998). Finally, and most notably, PXR and CYP3A are induced by similar xenobiotic agents. In general, drugs known to induce CYP3A *in vivo* are also able to activate PXR within the same species (Jones et al., 2000).

PXR is still considered an orphan nuclear receptor with endogenous ligands yet to be identified. However, it is becoming increasingly clear that PXR is a critical regulator of CYP3A gene expression. Thus, the activation of PXR can be used as a successful predictor of CYP3A induction in several species.

1.2.4 CYP2C11

CYP2C11 is predominantly expressed in the liver of adult male rats. Although there is no direct human corollary for this isoform, CYP2C11 has been implicated in the metabolism of several substances. Some of these include: tamoxifen (Mani et al., 1993), sildenafil (Warrington et al., 2002), and

the 16α- and 2α-hydroxylation of testosterone (Waxman, 1984b), among others. CYP2C11 accounts for up to 50% of the total hepatic P450 content in the male rat (Morgan et al., 1985) while it is virtually undetectable in female rats. This sexual dimorphism in enzyme expression is one of the hallmarks of CYP2C11 in the rat. Because of this condition, the rat is often chosen as an animal model for the study of the regulation of sexual dimorphisms in drug metabolism. Although humans do not exhibit such large inter-gender differences in the levels of drug metabolizing enzymes, the investigation of these exaggerated differences in rats will nevertheless add to a foundation of knowledge that will help explain other aspects of drug metabolism that are studied in the rat.

It is well established that GH is the mediating factor responsible for the sex-dependent expression of CYP2C11 (Kato et al., 1986; Legraverend et al., 1992; Waxman et al., 1991). More specifically, it was found that sexually distinct ultradian rhythms of GH secretion regulated the gender-specific expressions of rat hepatic monooxygenases. In the male rat, GH pulses are released in periods that are between 3.5 and 4 hours apart (Jansson et al., 1985; Tannenbaum and Martin, 1976). GH is nearly undetectable during the "between peaks" period. Following this initial finding, it was determined that it was not the number of pulses of GH, nor amplitude that regulated the expressions of hepatic P450s. Rather, it was the interpulse period that dictated the expression.

In order for male liver cells to express CYP2C11, a minimum "off time" is required during which no detectable levels of GH are present in circulation (Waxman et al., 1991).

The suppressive effects of CsA on CYP2C11 are similar to that of CYP3A2. However, CYP2C11 is not as responsive to CsA treatment as CYP3A2. For instance, CYP3A2 is suppressed at a CsA concentration of 5 mg/kg/day and higher, whereas CYP2C11 was only significantly suppressed at 15 mg/kg/day or higher (Brunner et al., 1998a; Brunner et al., 2000). Since CYP2C11 is largely regulated by GH, the suppression of this isoform caused by CsA could potentially be mediated through GH. However, our investigation into this event revealed that the suppressions of CYP2C11 and CYP3A by CsA was determined not to be caused by an alteration of GH levels alone (Lu et al., 2003a).

1.3 GROWTH HORMONE

GH is a 22 kD single chain peptide that is synthesized in somatotropic cells of the anterior pituitary. Its structure and gene organization are closely related to prolactin and placental lactogens. Unlike other pituitary hormones, GH lacks specific target sites. Because of this, it can exert a wide range of physiological and metabolic effects on target tissues. Although the chief function of GH is to promote postnatal longitudinal growth, GH is also known

to regulate lipid, carbohydrate, nitrogen and mineral metabolism within the cell (Davidson, 1987).

In addition to these basic functions, GH also plays an integral role in the immune system. Human T and B lymphocyte cell lines were shown to produce and secrete immunoreactive growth hormone (irGH) (Kao et al., 1992). This irGH molecule appeared to be *de novo* synthesized and their molecular size was similar to that of pituitary GH as well as irGH secreted by peripheral blood lymphocytes. Indirect evidence in rats also implicates a role for GH in the immune system. Rat mononuclear leukocytes also possess specific receptors for growth hormone releasing hormone (GHRH). One study shows that the binding of I^{125}-labeled GHRH to spleen and thymic cells was saturable with a high affinity for GHRH (Guarcello et al., 1991). In addition, low basal levels of specific GHRH mRNA were detected in the cytoplasm of rat leukocytes (Weigent and Blalock, 1990). It was shown that these lymphocytes produce an immunoreactive GHRH that is similar to hypothalamic GHRH with respect to bioactivity, antigenicity, and molecular weight, suggesting that GH release may be regulated by an immunomodulatory loop. Furthermore, detrimental effects on the immune system were demonstrated when GH was depleted *in vivo*. Hypophysectomized and sham-operated adult male rats were given either daily GH or saline treatment to investigate the effect of restoring GH. The hypophysectomized rats exhibited reduced antibody synthesis, DTH reactions,

NK cytotoxicity, IL-2 (or IL-4) production, body and organ weights, RBC counts, packed cell volumes, and hematocrits compared to sham-operated controls (Exon et al., 1990). GH treatment restored antibody and IL-2 production, thymic weights, and partially restored DTH reactions. This study was pivotal in implicating the pituitary in maintaining normal immune functions, particularly with respect to the production of GH.

The synthesis and release of GH is controlled by two hypothalamic peptides: growth hormone-releasing hormone (GHRH) and somatostatin, or somatotropin release-inhibiting factor (SRIF) (Tannenbaum, 1991). GHRH binds to G-protein coupled receptors on the surface of somatotrophs where GH is stored. Upon binding, the intracellular concentrations of cAMP and/or Ca^{2+} levels increase, which in turn, stimulates the secretion of GH (Mayo, 1992). Experimental data in the male rat have shown that alterations in the hypophyseal portal blood levels of GHRH and SRIF correspond to the pulsatile secretion of GH (Plotsky and Vale, 1985). More recently, a class of synthetic molecules called GH-releasing peptides (GHRP) have been shown to be capable of stimulating GH release. In man and rat, GHRP-1 is more potent than GHRH at eliciting GH release (Bowers, 1998).

The physiological actions of GH are mediated through a peptide called insulin growth factor-1 (IGF-I). Once GH is released into circulation, serum GH then binds to growth hormone receptors (GHRs) on the surface of cells on

target tissues, such as liver, muscle, and bone. This elevates the serum concentrations of IGF-1, which then acts upon these target tissues to initiate growth. Extremely high levels of IGF-I in serum help to trigger a negative feedback loop that inhibits GHRH and GH release (Ceda et al., 1987). The IGFs, IGF receptors, and IGF binding proteins (IGF-BP) are part of a family of cellular modulators that aid in the regulation of growth and development. The IGFs consist of two structurally related peptides: IGF-I and IGF-II. Both forms are expressed ubiquitously. IGF-I is secreted by many tissues with the secretory site generally determining its actions. Most IGF-I is secreted by the liver and is transported to other tissues, acting as an endocrine hormone. IGF-I is critical in growth and skeletal development as was demonstrated through studies with gene-knockout mice. GHR/BP knockout mice exhibited severe postnatal growth retardation, proportionate dwarfism, absence of the GHR and GH binding protein, greatly decreased serum IGF-I and elevated serum GH concentrations (Zhou et al., 1997). Thus, even in the presence of GH, normal growth functions do not take place when the IGF-I receptor is depleted, indicating that the binding of IGF-I to its receptor is essential in maintaining growth. In fact, in another knockout model, a knockout of the IGF-1 gene or the IGF-1 receptor gene reduces the size of mice by 40-45% (Accili et al., 1999). The absence of the IGF-I receptor in mice at birth is lethal due to impaired

development of the diaphragm and intercostal muscles leading to respiratory failure (Liu et al., 1993).

GH secretory patterns are known to regulate the sexual dimorphism in hepatic drug metabolism. The most apparent example is the expression of CYP2C11 in male rats and conversely CYP2C12 in females. In the male rat, GH is released in 250-300 ng/ml bursts and in periods that are between 3.5 and 4 hours apart (Jansson et al., 1985; Tannenbaum and Martin, 1976). During the interpeak period, levels of GH are nearly undetectable. This facet of the male-type release profile turned out to be pivotal. It was determined that neither the number of pulses of GH nor the amplitude was the critical factor regulating the expressions of hepatic P450s. Rather, it was the interpulse period that dictated the expression. In order for male liver cells to express CYP2C11, a minimum "off time" of approximately 2.5 hours is required during which no detectable levels of GH are present in circulation (Waxman et al., 1991). In fact, in male rats that had depressed overall levels of GH caused by MSG treatment, the GH peaks were significantly lower in concentration than in normal male rats. However, they still possessed typically male hepatic P450 expression, indicating that neither the amplitude nor the total GH content is crucial, but rather the presence of the ultradian rhythm (Shapiro et al., 1989). Conversely, when GH is secreted in a continuous pattern characterized by short intervals of low amplitude pulses, the expression of predominantly female isoforms,

CYP2C12, CYP2C7, and CYP2A1 is present. When adult male rats were hypophysectomized and re-administered GH by continuous infusion (indicative of the female secretion profile), they began to express the female-specific CYP2C12 (Pampori and Shapiro, 1999).

GH's actions are mediated through activation of the GH receptor (GHR), a member of the cytokine/hematopoietin receptor superfamily. Through the use of x-ray crystallography, size exclusion chromatography, and calorimetry methods, it was postulated that one GH molecule binds two GHRs to form a dimer complex (Cunningham et al., 1991). The membrane-bound GHR lacks intrinsic tyrosine kinase activity. However, GH promotes the association of GHR with JAK2, activates JAK2, and stimulates the phosphorylation of tyrosines within the GHR and JAK2 complex (Argetsinger et al., 1993). JAK2 not only phosphorylates tyrosines within itself, but also on the associated GHR. Following this event, the phosphorylated entities form high-affinity binding sites for a variety of signaling proteins including signal transducers and activators of transcription (Stats). Stat proteins then bind to specific DNA sequences in the nucleus to initiate transcriptional activation of target genes.

1.4 PROLACTIN

1.4.1 Background

Prolactin (PRL) is a polypeptide hormone that is synthesized and released by lactotrophs located in the anterior pituitary. However, evidence also shows that PRL is released by lymphocytes as well (Montgomery et al., 1990; Russell, 1989). It is well established that lactotrophs in the pituitary have spontaneously high secretory activity. Therefore, PRL secretion is under constant inhibitory control that is exerted by the hypothalamus. Studies have shown that when the pituitary is surgically disconnected from the medial basal hypothalamus, PRL levels gradually increase over several days until a plateau occurs (Bishop et al., 1972; Kanematsu and Sawyer, 1973). In addition, complete transplantation of the anterior lobe of the pituitary to the kidney capsule, which has no neural connection to the hypothalamus, results in a high spontaneous rate of PRL secretion (Everett, 1954; 1956). Thus, the release of PRL *in vivo* is tightly controlled by the hypothalamus in an inhibitory manner.

PRL is involved in a variety of biological functions in mammals including lactation, reproduction, and even osmoregulation (Nicoll, 1980). PRL also induces the production of ornithine decarboxylase (ODC) in various rat tissue systems including the neonatal brain (Roger et al., 1974), prostate (Rui and Purvis, 1987), spleen, thymus (Russell and Larson, 1985), and cultured

mammary tumor cells (Frazier and Costlow, 1982). ODC is a rate-limiting enzyme in the biosynthesis of vital polyamines needed for the stabilization of polynucleic acids and ribosome structure. Thus, in addition to its numerous roles in living systems, PRL indirectly aids in cell proliferation as well.

PRL's role in immune function has been observed as early as 1978 when it was discovered that hypophysectomized rats have diminished immune function in regards to both humoral and cell-mediated immunity (Nagy and Berczi, 1978). When bromocriptine (a PRL antagonist) was administered to normal rats, it suppressed the immune system in a similar fashion, thus confirming PRL's role in the maintenance of immune function (Nagy et al., 1983). PRL is also associated with numerous other aspects of the immune system. For instance, PRL receptors were found on normal T- and B-cells (Cardon et al., 1984), as well as Nb2 cells (Shiu et al., 1983). The administration of PRL to rat splenic lymphocytes induces the expression of interleukin-2 receptors on the surface of lymphocytes *in vitro* (Mukherjee et al., 1990). Furthermore, PRL and CsA compete for binding sites on lymphocytes (Cardon et al., 1984). CsA and PRL have been shown to interact directly. In one report, acute CsA treatment to rats resulted in a four-fold increase in serum PRL concentration within one hour after administration (Cardon et al., 1984). This effect held true only for the 12 μg/100 g body weight dose, although significant increases were also observed for the 0.12 and 1.2 μg/100 g body

weight doses. This induction was reversed with the subsequent administration of bromocriptine. Others have demonstrated a direct effect of CsA on the circulating levels of PRL. In one of the first studies to examine the effects of CsA on the pulsatile release of PRL, researchers discovered that subcutaneous injections of low-dose CsA to rats with an ectopic pituitary graft decreased the absolute amplitude and mean values of PRL release as compared to rats with ectopic pituitary grafts alone (Esquifino et al., 1996). CsA has also been shown to significantly repress PRL-induced polyamine biosynthesis in the spleen and thymus of rats (Russell and Larson, 1985), as well as the kidney and adrenal gland (Cardon et al., 1984), thus providing another possible avenue for interactions between CsA and PRL.

The intracellular signaling of PRL has been elucidated in recent years. Typical of endocrine factors, there appears to be more than one signaling pathway induced by PRL. In the Nb-2 rat lymphoma cell line, PRL induced both rapid activations of both c-*jun* N-terminal kinases (JNKs) (Schwertfeger et al., 2000) and Janus kinase 2 (JAK2) (Campbell et al., 1994). In bone marrow-derived murine pro-B lymphoid cells, PRL induced rapid tyrosine phosphorylation of JAK2 (Dusanter-Fourt et al., 1994). Further evidence for the involvement of the JAK/Stat pathway was shown in studies in HC11 cells, a mammary epithelial cell line. PRL treatment specifically activates Stat5,

suggesting that Stat5 is involved in the downstream intracellular signaling events triggered by the PRL receptor (Ali and Ali, 1998).

1.4.2 Bromocriptine

Bromocriptine (BRC) is an ergot alkaloid that is a specific dopamine receptor agonist (Figure 1.4.2). BRC drastically reduces the secretion of prolactin (PRL) from the pituitary, and thus overall circulating levels. Due to BRC's ability to rapidly suppress PRL secretion, it has been successfully used to treat a variety of conditions including: hyperprolactinemia (Floersheim-Shachar and Keller, 1977), pituitary adenomas (Scamoni et al., 1991), acromegaly (Wass et al., 1977), and early stage Parkinson's disease (Kondo, 2002; Parkes et al., 1976).

Few studies regarding BRC and the P450 system have been conducted. Initially, CYP3A was implicated in the hydroxylation of BRC at the proline ring of the cyclopeptide moiety (Peyronneau et al., 1994a). However, several years later, a study using the perfused rat liver model demonstrated that a CYP3A inhibitor, troleandomycin, had virtually no effect on the metabolism of BRC (Matsubayashi et al., 1997). The authors did not exclude the potential for the involvement of CYP3A on the metabolism of BRC, but did hypothesize that free radical scavenging in the liver may account for part of the elimination process of BRC from the liver. Another study performed in rat liver

microsomes showed an inhibition of P-450 dependent monooxygenase activity caused by BRC (Moochhala et al., 1989). Overall, there appears to be a relationship between BRC and the P450 system, although the connection is not clearly defined in either *in vivo* or *in vitro* conditions.

BRC has been tested as an adjuvant to CsA therapy in order to reduce potentially toxic CsA concentrations. In one study in humans, the combination of BRC along with CsA therapy in the first three months following surgery significantly reduced the incidence of renal transplant rejection when compared with the traditional triple-drug regimen for immune suppression, which includes cyclosporine, azathioprine and steroids (Yongjin et al., 1997). These results were supported by studies in rats that demonstrated that adjunct administration of BRC is also successful in reducing organ rejection when used in conjunction with low-dose CsA. Rats with transplanted pancreas allografts had significantly longer graft survival times when dosed with both drugs concomitantly as compared with either drug alone (Ferrero et al., 1987). Furthermore, the addition of BRC microcapsules to existing low-dose CsA therapy is beneficial in the treatment of adjuvant arthritis. Rats administered BRC in combination with CsA not only experienced decreased limb thickness due to arthritis, but also, only 10% of a regular CsA dose was required (Neidhart, 1996). Thus, combination therapy with BRC could potentially

decrease organ toxicities associated with high effective doses of CsA in patients.

1.5 JAK/STAT PATHWAY

1.5.1 Background

Once a cytokine or hormone binds to a cell surface receptor, it induces conformational changes that trigger a cascade of intracellular events. In the case of GH, GH receptors are located on the surface of liver, kidney, gastrointestinal tract, adipose, heart, lung, and muscle cells (Mathews et al., 1989; Tiong and Herington, 1991). However, these receptors are single chain transmembrane proteins that lack intrinsic kinase activity. Due to this deficiency, cellular events could not be initiated without the help of protein kinases, such as Janus kinases (JAKs). The conformational changes that take place within the receptor activate JAKs and induce them to autophosphorylate and/or transphosphorylate and in turn phosphorylate the receptor. The phosphorylated receptor serves as a docking site for signal transducers and activators of transcription (Stats), which dimerize and translocate to the nucleus to initiate gene transcription of specific proteins.

1.5.2 Janus Kinase (JAK)

JAKs are a family of protein kinases that include JAK1, JAK2, JAK3, and TYK2 that range in size from 110 kDa to 140 kDa (Aringer et al., 1999). There are seven regions of high homology within the JAK family, JH1-JH7. Amongst these domains, JAKs have a distinctive feature in that they contain a kinase domain (JH1) and a pseudokinase domain (JH2). Because of this dual-sided structure, the "Janus" portion of JAK's name was derived from the two-faced Roman god of gates and passages. The pseudokinase domain lacks several residues critical for activity and is thus considered to be catalytically inactive when expressed alone. However, when analyzed together, the JH2 domain appears to be necessary for JH1 catalytic activity (Yeh et al., 2000) and negative regulation of JAK2 (Saharinen et al., 2003). The remainder of the domains, JH3-JH7, has been implicated in receptor association function.

JAK1, JAK2, and TYK2 are ubiquitously expressed while JAK3 is found only in cells of hematopoeitic lineage (Tortolani et al., 1995). Collectively, these kinases can be differentially activated by nearly all cytokines found in the body (Ihle, 1995). These cytokines include growth hormone (Argetsinger et al., 1993) and prolactin which both activate JAK2 (Campbell et al., 1994; DaSilva et al., 1994; Dusanter-Fourt et al., 1994). Other cytokines such as ones included in the interferon (IFN) family, virtually all interleukins (IL), ethrythropoietin, thrombopoietin, epidermal growth factor,

hepatocyte growth factor, and platelet-derived growth factor are known to activate various members of JAK (reviewed in (Kisseleva et al., 2002).

Once cytokines bind to their respective cell surface receptors, these receptors undergo conformational changes that attract JAKs to come within close proximity of each other (Remy et al., 1999). This event allows for the trans-phosphorylation action and subsequent activation of JAK proteins. The GH receptor (GHR) was the first to be identified in the cytokine receptor superfamily (Cosman et al., 1990). When appropriate levels of circulating GH are present *in vivo*, one GH molecule binds to two GHR extracellular domains thus, leading to receptor homodimerization (Figure 1.5.3). This 2:1 ratio of GHR to GH was found to exist through X-ray crystallography (Cunningham et al., 1991). This homodimerization induces two JAK2 molecules to cross-phosphorylate each other and in turn phosphorylate the intracellular domains of the GHR (Carter-Su and Smit, 1998). To date, virtually all signaling pathways and downstream events that employ the use of the GHR require the involvement of JAK2 activity with the exception of GH-dependent calcium entry (Billlestrup et al., 1995).

JAK deficiencies, particularly in fetal development, have extremely detrimental effects. When JAK2-deficient mouse embryos were developed, the embryos expired around day 12 as a result of the absence of definitive erythropoiesis (Parganas et al., 1998). Mice lacking JAK3 display critical

immune defects that include low peripheral T-cell count, decreased proliferative response to IL-2, and a lack of natural killer cells (Yeh and Pellegrini, 1999). In humans, a deficiency in JAK3 leads to autosomal-linked severe combined immune deficiency (SCID). SCID is characterized by a block in lymphoid development thus leading to an increased susceptibility to infections. SCID patients with mutations in the JAK3 locus express little to no protein or non-functional forms as a result (Macchi et al., 1995; Russell et al., 1995).

1.5.3 Signal Transducers and Activators of Transcription (Stat)

One of the most appealing aspects of the JAK/Stat pathway lies in its simplicity. It involves a direct, linear pathway from the cell surface to gene expression (Figure 1.5.3). The Stat family of transcription factors was initially identified through efforts to identify the factors that mediated the activation of genes following interferon (IFN) binding (Darnell et al., 1994; Schindler and Darnell, 1995). Stats comprise a family of seven structurally and functionally related proteins including: Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6. The Stat protein responsible for mediating the physiological effects of GH is Stat5b (Waxman et al., 1995b).

JAKs are responsible for the tyrosine phosphorylation of Stats, which is responsible for the dimerization of Stats and subsequent translocation of the dimer complex to the nucleus (Ihle, 1996; Pellegrini and Dusanter-Fourt, 1997).

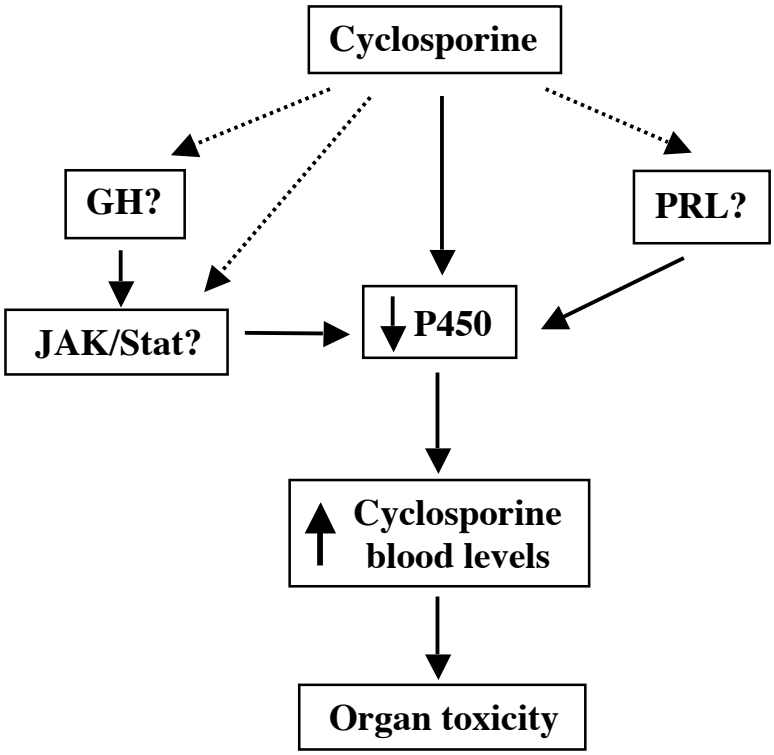
The homo- or heterodimerization of Stats is mediated through tyrosine phosphorylation of a carboxy-terminal site of the SH2 domain. Serine phosphorylation is necessary for the DNA binding and transactivation once inside the nucleus (Wen et al., 1995; Zhang et al., 1995). The activated Stats bind to specific DNA sequences to initiate transcription of target genes.

The effect of intermittent GH pulses on Stat5b disposition was investigated in both male and female hypophysectomized rats. In male rats, intermittent GH induced in tyrosine phosphorylation, nuclear translocation, and DNA-binding activity of Stat5 (Waxman et al., 1995b). More recently, the Stat5 form responsive to GH was identified as Stat5b (Yi et al., 1996). Since then, Stat5b was suspected of being the major, if not sole, protein mediating sex-specific effects of GH pulses in the liver.

While it is well-established that intermittent GH pulses induces the expression of Stat5b in the male rat, recent evidence suggests a direct connection between levels of Stat5b and the expression of CYP2C11, implicating Stat5b as an intracellular mediator of GH effects on P450 expression. The administration of ethanol to adult male rats resulted in a significant reduction of CYP2C11 in the liver and kidney along with a corresponding decrease in Stat5b and phospo-Stat5b (P-Stat5b) (Badger et al., 2003). Although the amounts of both Stat5b and P-Stat5b were reduced as a result of ethanol treatment, the ratio of Stat5b to P-Stat5b did not differ from

the control. Thus, it was hypothesized that ethanol's suppressive effect on CYP2C11 was attributed to lowering Stat5b levels and not through alteration of P-Stat5b through JAK2. However, in another study in prepubertal rats, supraphysiological levels of GH caused an increase in Stat5b, but no change in CYP2C11 expression (Choi and Waxman, 2000). This result was attributed to either a lack of responsiveness to GH in prepubertal rat liver or to the involvement of other factors present only in adult rats. These and other data combine to implicate a role for Stat5b in the expression of sexually dimorphic P450 enzymes in response to GH pulses.

1.6 Objectives



The aims of the current work were to 1.) investigate growth hormone as an intermediate in the suppression of P450s by cyclosporine, 2.) investigate the role of prolactin in cyclosporine-induced alteration of P450 enzymes, and 3.) examine the mechanistic effects of cyclosporine on the JAK/Stat pathway and the consequences of these modifications on JAK/Stat in the modulation of CYP3A1/2 and CYP2C11 in rat hepatocytes. The above illustration shows proposed interactions for the following studies in dashed arrows while established relationships are denoted with full arrows.

Figure 1.1 Structure and primary metabolites of cyclosporine

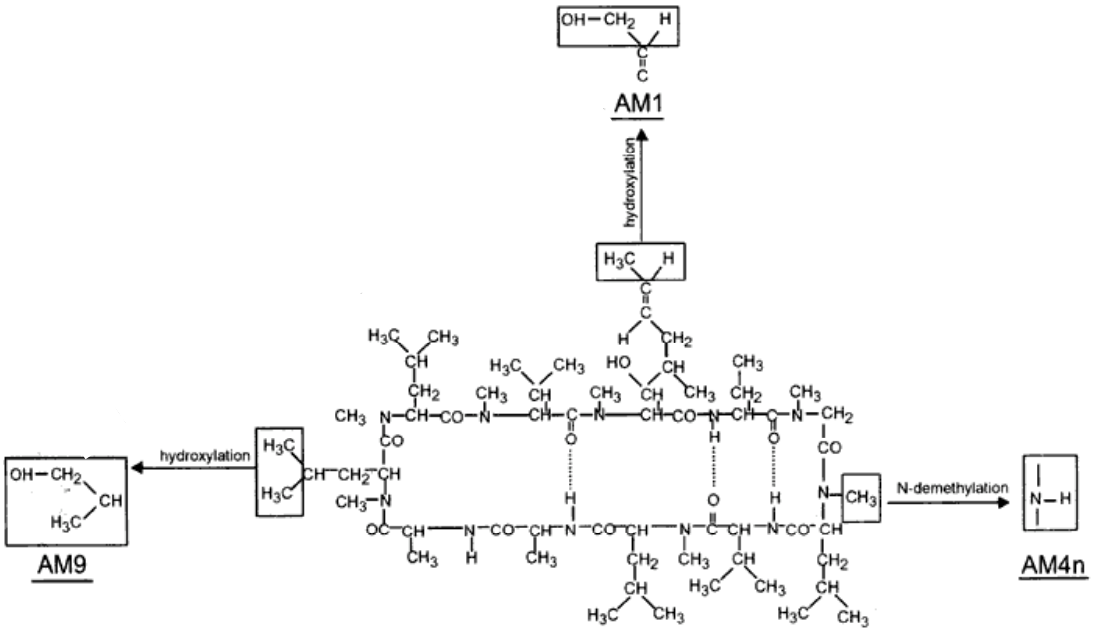


Figure 1.1

Structure of cyclosporine and the main metabolic reactions it undergoes.

Figure 1.2 P450 reaction cycle

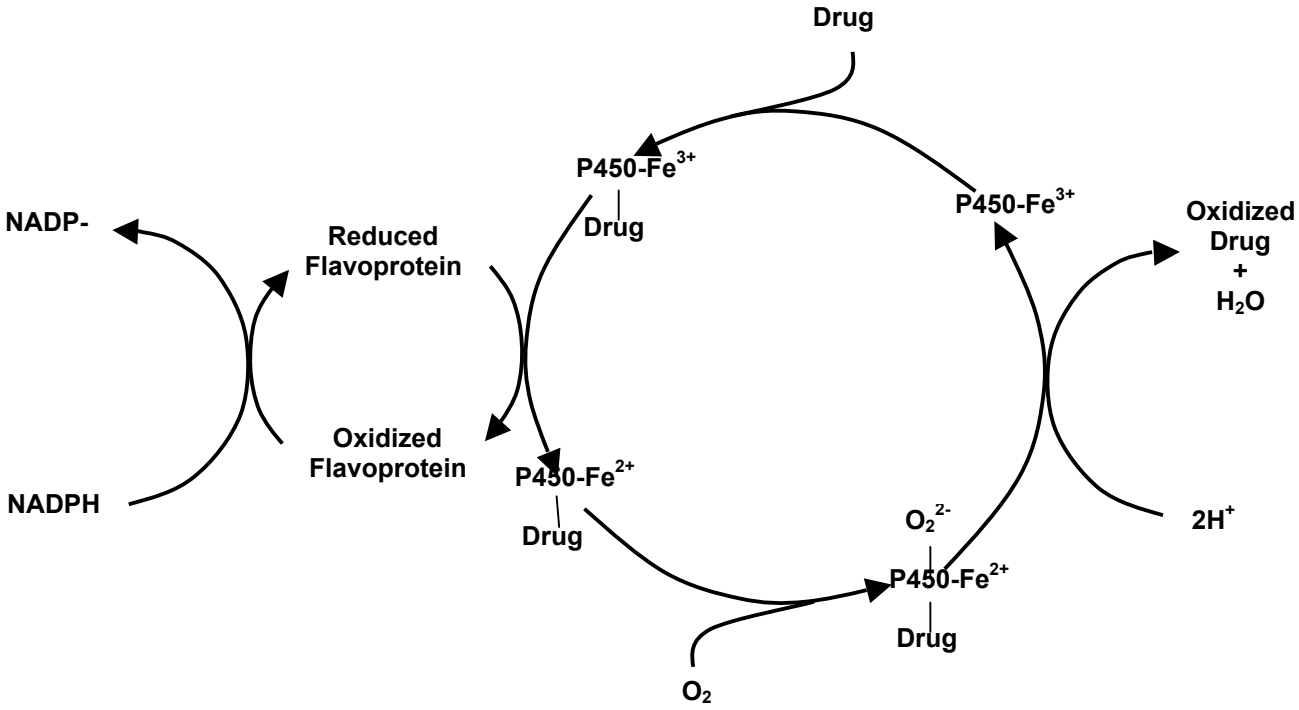


Figure 1.4 Structure of bromocriptine

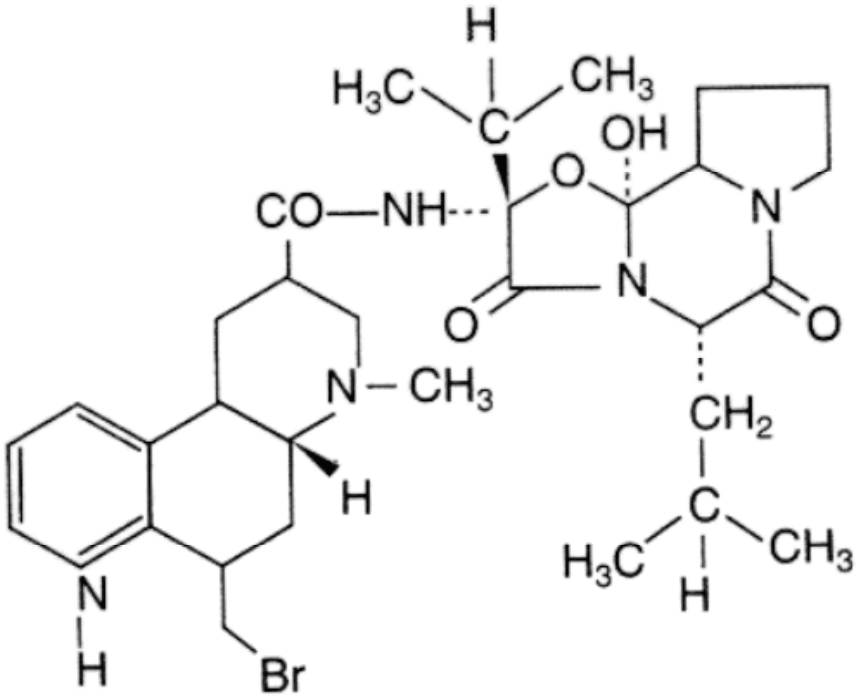


Figure 1.5 Cellular signaling

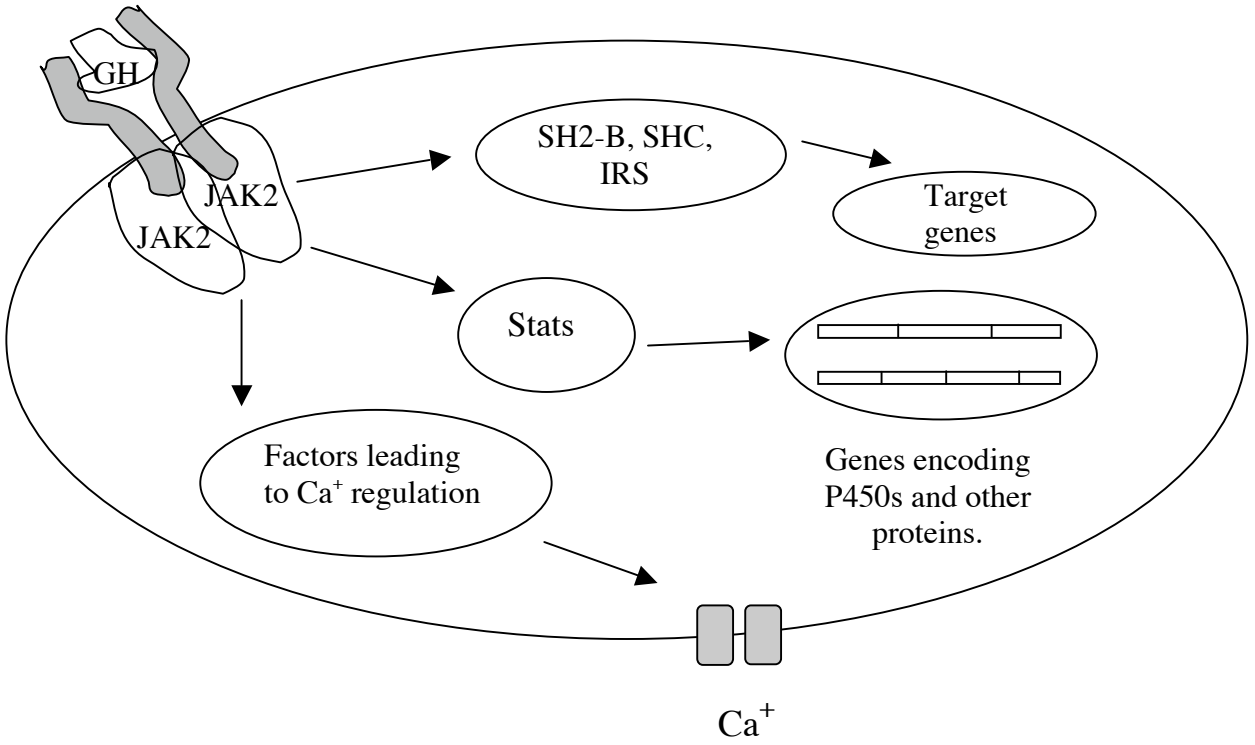


Figure 1.5

Schematic illustration of the JAK/Stat intracellular signaling pathway.

CHAPTER TWO

Suppression of Hepatic CYP3A1/2 and CYP2C11 by Cyclosporine is Not Mediated by Altering Growth Hormone Levels

2.1 INTRODUCTION

Immune suppression has been an effective avenue of treatment for several conditions including preventing organ transplantation rejection and autoimmune disease. One potent immunosuppressive drug cyclosporine (CsA), a cyclic undecapeptide of fungal origin, is often used as the drug of choice following organ transplantation. CsA is mainly used for the prevention of allograft rejection and for the prevention of graft-versus-host disease following a bone marrow transplant as well as for the treatment of arthritis (Tugwell et al., 1987). Although CsA is known to affect interleukins 3 and 4 (IL-3, IL-4), tumor necrosis factor-α (TNF-α), and B cells, it primarily imparts its immunosuppressive action by preventing interleukin-2 (IL-2) synthesis from activated T-cells (Bunjes et al., 1981).

Despite the effectiveness of CsA in suppressing the immune response, there exist several potentially harmful side effects, including nephrotoxicity, hepatotoxicity, and hypertension (Borel, 1990). Since CsA is not only a substrate, but also an inhibitor of CYP3A2, CsA can modify hepatic drug metabolism in rats following chronic therapy (Brunner et al., 1996b). CsA suppresses cytochrome P450 (CYP) protein expression, which could then hinder further metabolism of CsA (Brunner et al., 1996b; Cunningham et al., 1985). This cycle results in an accumulation of CsA ultimately leading to organ toxicity. More specifically, it was found that CsA suppressed hepatic CYP3A1/2 and 2C11 in a time-dependent and dose-dependent manner in animals (Brunner et al., 1998b; Brunner et al., 2000). Although this phenomenon has been readily demonstrated in the rat, CsA-induced changes in hepatic metabolism have yet to be identified clearly in humans.

Pituitary hormones are known to play a significant role in P450 expression in mammals. Unlike other pituitary hormones, growth hormone (GH) lacks specific target sites. Because of this, GH can exert a wide range of physiological and metabolic effects on target tissues. GH alters drug metabolism in the liver by influencing the regulation of P450 expression. From as early as 1973, the regulation of hepatic drug metabolism enzymes in the rat was shown to be dependent on the amount of GH present *in vivo* (Wilson, 1973). In determining sex-specific expressions of drug-metabolizing enzymes,

the pattern of GH secretion is more relevant than the actual amount of GH present. When GH is secreted in a pulsatile fashion, the male-specific P450 isoform CYP2C11, along with its corresponding steroid 16α- and 2α-hydroxylase activities, predominates (Morgan et al., 1985; Waxman et al., 1991).Other P450 isoforms including CYP3A2, CYP2A2, and CYP2C13 are also male-specific, but may not necessarily depend directly on intermittent GH pulses (Waxman et al., 1988b; Waxman et al., 1995a). On the other hand, if GH is secreted in a continuous pattern, this is indicative of a female secretion pattern. In this case, CYP2C12 prevails, while levels of CYP2C11 and CYP3A2 are low to nonexistent (MacGeoch et al., 1985; Waxman et al., 1985). Since P450 enzymes account for the majority of drug metabolism that takes place in a rat, any alteration of these enzyme levels could have a profound impact on the extent of drug metabolism and possibly lead to adverse pharmacological effects.

The purpose of this study was to investigate the role of GH in the modulation of drug-metabolizing enzymes by CsA. This could provide additional insight into the role of hormones in the regulation of drug metabolism and provide a clearer understanding of the physiological interactions that mediate the effects of CsA on P450 enzymes.

2.2 MATERIALS AND METHODS

2.2.1 Materials

CsA was generously provided by Novartis (East Hanover, NJ, USA) in the form of Sandimmune™ oral solution. The original dosage form was diluted in commercially available olive oil and stored in amber bottles to limit light exposure. The CsA vehicle was the same commercially available olive oil. Purified rat GH was generously provided by the National Hormone & Pituitary Program under the National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK, Torrance, CA, USA) and solubilized in 0.01M $NaHCO_3$. GH vehicle consisted of 0.01M $NaHCO_3$. The anesthetic mixture consisted of a 1:1:1 (v:v:v) ratio of ketamine (100 mg/ml), xylazine (20 mg/ml), and acepromazine (10 mg/ml). Ketamine and xylazine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acepromazine was purchased from Fort Dodge Laboratories, Inc. (Fort Dodge, LA, USA). Rat CYP3A1/2 and CYP2C11 selective polyclonal antibodies were purchased from Gentest Corporation (Woburn, MA, USA). Rat CYP3A1/2 antibody was isolated from goats that were immunized with CYP3A2 purified from rat liver. Due to the polyclonal nature of the antiserum, two indistinguishable bands (CYP3A1 and CYP3A2) are detected and is therefore collectively referred to as CYP3A1/2. The microsomal standard used for the relative quantitation of CYP3A1/2

protein was composed of phenobarbital-treated rat liver microsomes. Rat CYP2C11 antibody is also polyclonal and raised in goats, however the cross reactivity with CYP2C13 can be readily distinguished from CYP2C11 based on mobility. CYP2C11 standard was composed of untreated male rat liver microsomes. The horseradish peroxidase-conjugated rabbit anti-goat (secondary P450 antibody) was purchased from ICN Pharmaceuticals, Inc. (Aurora, OH, USA). Rat growth hormone enzyme immunoassay (EIA) kits were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA) and were manufactured by Spi-Bio (Massy Cedex, France). All urine and serum creatinine assay slides were manufactured by Johnson & Johnson's Ortho-Clinical Diagnostics division (Rochester, NY, USA).

2.2.2 Animals

All procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas at Austin and are in accordance with the guidelines established by the National Institutes of Health for the humane treatment of animals. Nine- to ten-week old, male Sprague Dawley rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). During the study, all rats were kept in a twelve-hour light/dark cycle environment with free access to standard rat chow (Harlan, Indianapolis, IN, USA) and deionized water. Food consumption and body weight were measured daily.

Rats were randomly assigned to one of six groups (N=6 each). The groups are denoted: 14 CsA, 14 CsA V, 14 GH/CsA, 14 GH/CsA V, 14 GH V/CsA, and 14 GH V/CsA V (V = vehicle). Animals receiving GH were administered twice daily subcutaneous doses of 120 ng/g body weight of rat GH according to previous reports of successful supplementation of GH (Waxman et al., 1991). The GH vehicle was administered in the same fashion as GH at 1 ml/kg. CsA administration consisted of once daily subcutaneous injections of a 15 mg/kg dose and CsA vehicle was also administered once daily at a concentration of 1 ml/kg.

2.2.3 Blood collection

On the second to last day of dosing, all animals underwent jugular cannula implantation surgery to allow for passive blood collection according to the method of Waynforth & Flecknell (Waynforth and Flecknell, 1992). On the last day of dosing for each group, rats were placed into standard rodent metabolic cages for urine collection. Following this 24-hour period, 0.1 ml blood samples were collected once every 15 minutes for a six-hour period via the in-dwelling jugular cannula. After collection, the blood was allowed to clot on ice and was centrifuged at 9000 x *g* for 5 minutes at 4° C. Serum was harvested immediately following each spin and stored at –80° C until time of assay.

2.2.4 Microsome isolation

Upon sacrifice of animals, the liver was immediately excised. Liver microsomal isolation was achieved with the use of a previously described method of differential centrifugation (Coon et al., 1978a) and kept at 4°C during the entire preparation. Liver tissue was ground in three volumes of Tris chloride buffer consisting of 0.1 M EDTA and 0.15 M potassium chloride using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA, USA). Samples were then centrifuged at 9,000 x *g* for 20 minutes at 4°C. The supernatant was collected and centrifuged at 550,000 x *g* for 17 minutes at 4°C. The supernatant was discarded and the pellet resuspended in sodium pyrophosphate buffer containing 0.1 M EDTA at pH 7.4. The suspension was then homogenized and afterward centrifuged at 550,000 x *g* for another 17 minutes at 4 °C. The supernatant was discarded and the pellet washed and homogenized in Tris buffer containing 20% glycerol for storage. The microsomes were then stored at -80°C until analysis.

2.2.5 Gel electrophoresis and immunoblot analysis

Gel electrophoresis was performed using an SDS-PAGE 8% polyacrylamide separating gel as previously described (Laemmli, 1970). Protein on the gel was then transferred to nitrocellulose sheets by a prior described method (Schnier et al., 1989a). After transfer of proteins, the

nitrocellulose sheets were blocked with 3% non-fat dry milk (NFDM) in tris-buffered saline (TBS) at room temperature. Detection of putative proteins was achieved with goat anti-rat IgG (in a 1:2000 dilution) immunoreactive to the specific P450 enzyme of interest in 3% NFDM and then rabbit anti-goat horseradish peroxidase (in a 1:2000 dilution) also in 3% NFDM at room temperature. Transitional washes using TBS and 0.05% Tween 20 in TBS were done according to previously described procedures (Schnier et al., 1989a). Immune complexes for CYP3A1/2 and CYP2C11 were detected with an NEN chemilumnescence reagent kit as described by the manufacturer (New England Nuclear Life Science Products, Boston, MA, USA). Blot densities were measured using a flatbed scanner (Microtek, Hsinchu, Taiwan) and analyzed on a Dell PC computer using the Kodak 1D image analysis software, version 3.5 (Eastman Kodak Co., Rochester, NY, USA).

2.2.6 Testosterone Hydroxylation Assay and HPLC assay

Liver samples for the testosterone hydroxylation assay were performed as previously described (Brunner et al., 1996b). In brief, 200 μg of liver microsomal protein was added to 0.02M potassium phophate buffer (pH 7.4), regeneration system, and water. The samples were then incubated with 250 μM testosterone for 3 minutes and with glucose-6-phosphate dehydrogenase for 15 minutes under the same conditions. The reaction was quenched with

dichloromethane and 11α-hydroxyprogesterone was added as the internal standard. Once the organic phase was transferred and evaporated, they were dissolved in methanol and stored at 4° C for no longer than 1 month before use.

Separation and detection of testosterone and metabolites were performed on a Shimadzu HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) equipped with an automatic injection system, dual solvent delivery pumps, a system controller, and a variable visual/ultraviolet wavelength detector. The column (LC-18) was kept at a constant temperature of 40° C and a wavelength of 238 nm was used to detect the analytes. Peak areas of corresponding hydroxylation metabolites were measured and compared to peak areas of the internal standard within the same run.

2.2.7 Creatinine Clearance

Serum creatinine was evaluated using Vitros creatinine DT slides on the Vitros DT60 II chemistry system (Rochester, NY, USA). The analysis is based on the hydrolysis of creatinine to form ammonia. Once ammonia is formed and creates a blue color, the intensity of the color is detected by a photodetector located within a fiber-optic reflection system and compared to a blank ammonia slide. The concentration of sample is calculated based on these measurements and the calibration measurements.

Urine creatinine measurements were calculated based on a similar system as the serum creatinine with the exception that all components were contained in one slide. Creatinine clearance was calculated as follows:

Clcr = (urine creatinine/serum creatinine) X urine flow rate

Values were expressed in units of μl/min/100 g body weight.

2.2.8 rGH Enzyme Immunoassay

The enzyme immunoassay used to determine GH levels was manufactured by Spi-Bio (Massy Cedex, France). The principle of the assay is based on the competition between unlabelled rat GH and acetylcholinesterase bound to rat growth hormone tracer. The yellow color formed by exposing the sample and acetylcholinesterase to an enzymatic substrate (and chromagen) for acetylcholinesterase was measured spectrophotometrically. The limit of detection for this assay was 0.5 ng/ml.

2.2.9 CsA Monoclonal Whole Blood Assay

Levels of CsA in blood were measured using the TDxFLx® system (Abbott Laboratories, Abbott Park, IL, USA). The system uses fluorescence polarization immunoassay (FPIA) technology as the basis for detecting CsA in blood. In brief, the competitive binding assay involves a tracer-labeled antigen, antibody, and the sample blood. In reference to sensitivity, the CsA monoclonal whole blood assay can detect CsA levels with 95% confidence for samples

containing ≥25 ng/ml of CsA. Assay precision was determined by Abbott Laboratories to be <4% CV.

2.2.10 Data Analysis

Densities from Western blots were compared with standard microsomal proteins and expressed as a percentage. Mean peak quantity, mean peak amplitude, and mean peak duration determinations were calculated using the ULTRA analysis program (Van Cauter, 1988). In brief, the general principle of the program relies on the elimination of all peaks that do not meet the threshold requirements for a significant pulse. The measurements used are based on a comparison of the increment of the preceding nadir and the decrement of the preceding peak to a multiple of the intra-assay coefficient. Pulses that do not exceed the threshold criteria are eliminated from the series. One-way ANOVA and *a priori* means comparisons tests were employed using respective vehicle groups as the control with the aid of the SuperANOVA statistical program (Abacus Concepts, Inc., Berkeley, CA, USA). Data are presented as mean ± standard error. When the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$), the differences were then considered to be statistically significant.

2.3 RESULTS

2.3.1 Effect of concomitant administration of rGH and CsA on hepatic P450 levels

Figure 2.1 represents hepatic CYP3A1/2 and CYP2C11 protein expression after 14 days of dosing as determined by Western blotting. The results from rats treated for 14 days revealed a significant decrease in CYP3A1/2 and CYP2C11 levels in the CsA group as compared to the vehicle control group ($p=0.04$; $p=0.004$, respectively), which is in agreement with previous reports from our lab (Brunner et al., 2000). Similarly, when CsA was administered in combination with GH vehicle, both isoforms were markedly depressed when compared with GH vehicle/CsA vehicle treatment. When exogenous GH was given with CsA, levels of CYP3A1/2 and CYP2C11 were not suppressed as compared to the GH/CsA vehicle group. Additionally, when administered concomitantly with CsA vehicle, excess GH caused a significant decrease in CYP3A1/2 and CYP2C11 expression ($p=0.01$; $p=0.007$, respectively). Figure 2.5 depicts a representative Western blot to illustrate the relative differences in densities between the aforementioned groups.

2.3.2 Effect of concomitant administration of rGH and CsA on hepatic P450 activity measured by testosterone hydroxylation assay and HPLC

The formation of 6β-hydroxytestosterone (6β-OHT) correlates primarily with the activity of CYP3A2 (Waxman et al., 1985; Waxman et al., 1983), and to a lesser extent with CYP3A1 (Sonderfan et al., 1987). HPLC analysis following testosterone hydroxylation assay of liver microsomes showed a significant decrease in the production of 6β-OHT ($p=0.0001$) in the group receiving CsA only as compared to the group receiving CsA vehicle only, indicating a reduction in the overall activity of CYP3A1/2 (figure 2.2). This finding supports the suppression in protein levels found with Western blotting. Significantly lower quantities of 6β-OHT were detected in all groups administered GH (GH/CsA V, GH/CsA) versus the respective GH vehicle groups ($p=0.026$; $p=0.0001$), thus confirming that superphysiological expression of GH decreases CYP3A1/2 activity.

The formation of 16α-OHT and 2α-OHT correspond to the activity of CYP2C11 (Cheng and Schenkman, 1983; Waxman, 1984a). Since the pattern of results was nearly identical for the formation of these two metabolites, only the results for 2α-OHT are represented in graphical form. Following 14 days of treatment with CsA, activity levels of CYP2C11 were lowered significantly as compared with CsA vehicle treatment. CsA treatment alone and in conjunction with GH vehicle also dramatically decreased the formation of 2α-OHT

(p=0.0001; p=0.0001, respectively), as well as 16α-OHT (p=0.0001; p=0.0001, respectively). Interestingly, administration of GH (as compared to GH vehicle) significantly lowered the formations of 2α- and 16α- OHT, irrespective of the concomitant drug (CsA or CsA vehicle).

2.3.4 Effect of concomitant administration of rGH and CsA on renal function parameters

Urine volume collected over the 24-hour period was compared as a measure of kidney function (table 1). CsA-treated rats had a nearly 2-fold greater volume of urine output as compared with the CsA vehicle-treated rats (p=0.02). Similarly, the CsA-treated rats given GH vehicle had a 2.3-fold increase in urine volume when compared with CsA-treated rats given GH vehicle. However, when CsA-treated rats were administered GH, the urine flow volume was significantly decreased from the CsA/GH vehicle group (p=0.038).

Serum creatinine levels and creatinine clearance rates were analyzed as an estimation of glomerular filtration rate (table 1). Serum creatinine was only slightly higher in the GH/CsA group as compared with the GH/CsA vehicle group (p=0.05). No other differences in serum creatinine levels were detected for any of the other groups. In addition, no significant differences in creatinine clearance, due to either administration of GH or CsA, were detected.

2.3.5 Circulating serum growth hormone levels

Figure 2.3 shows the area under the curve (AUC) for all groups. The groups that were administered two injections a day (regardless of the agents injected) generally had higher AUCs due to the combination of both higher frequency of pulses and a higher basal level of secretion. No statistically significant differences were observed between respective treatment groups. Representative six-hour GH secretion profiles from each group are presented in figure 2.4. GH release profile parameters including mean peak amplitude, mean peak number, and mean peak duration are shown in table 2. No statistically significant differences were detected between treatment groups and their respective controls.

2.3.6 CsA Blood Levels

The concentration of CsA in blood for animals administered GH and CsA was slightly higher, but not statistically different from animals injected with GH vehicle and CsA ($p=0.175$). This was the only comparison performed since no other comparisons were useful from a statistical standpoint.

2.4 DISCUSSION

The primary aim of the present study was to determine if GH is the main intermediate through which the chronic suppression of P450 by CsA

occurs in the liver of rats. With the pituitary intact, the GH secretory profile is still present and suppression of normal levels of P450 enzymes can be readily detected. The relationship between CsA, GH, and P450 enzymes was examined by introducing exogenous GH to intact male rats while concomitantly administering CsA. Select P450 isoforms' expressions and activities were analyzed as indicators of drug metabolism.

Because CYP3A1/2 contributes significantly to the metabolism of numerous xenobiotics (including CsA) that takes place in the rat liver and CsA is known to suppress this isoform, the expression and behavior of this isoform was of chief importance for this study. Previous studies show that when exogenous GH is introduced to intact male rats, CYP3A2 is significantly suppressed (Kawai et al., 2000; 2001). Our study confirmed that 14-day treatment with GH suppresses CYP3A1/2 protein expression. However, when CsA was administered in combination with GH, GH did not cause a significant suppression of CYP3A1/2 protein as compared to concomitant administration of CsA with GH vehicle. This masking effect was also evident when CsA failed to cause suppression when administered with GH and compared to administration with GH vehicle. Also, all groups receiving two injections per day experienced an overall decline in CYP3A1/2 protein expression, irrespective of the drug. This finding corresponds with the trend in GH levels represented by AUC calculations in this study. GH levels for groups receiving

two injections were generally higher than groups receiving only one. This could partly be due to the stress involved with the injection and is therefore unrelated to the drug admininistered. A previous study revealed a significant increase in GH following 10-11 days of only saline injection in adult male rats (Kant et al., 1983).

Since CYP3A1/2 catalyzes the production of both 6β-OHT and 2β-OHT, the quantities of both derivatives were measured to signify CYP3A1/2 activity. We showed that after 14 days of dosing, exogenous rat GH administered to normal rats suppressed both 6β- and 2β-OHT production. This result was evident despite co-administration with either CsA or CsA vehicle. Similarly, a previous study has reported depression of 6β-OHT levels after 7 days in intact male rat given GH in both intermittent pulses as well as in a continuous infusion (Yamazoe et al., 1986). Because the continuous presence of GH has been shown to decrease CYP3A2 activity in male rats and the long-term intermittent pulses used for this study resulted in a decrease in the activity of CYP3A1/2, this indicates that CYP3A1/2 may be responsive to an overall chronic increase in circulating GH levels. The data also show that GH and CsA have an additive suppressive effect on CYP3A1/2 activity as compared to CsA's suppressive effects alone. Since CYP3A2 is the main isoform responsible for the metabolism of CsA, the considerable decline in CYP3A1/2 activity as a result of CsA and GH combined will likely lead to higher

concentrations of CsA in circulation, thus leading to increased incidence of organ toxicity.

CYP2C11 is a sex-specific isoform whose expression is most directly regulated by the pulsatile secretions of the male GH pattern (Legraverend et al., 1992; Waxman et al., 1991). This concept was further validated when pulsatile secretions of GH via an external syringe pump as well as subcutaneous injections replaced the male-pattern GH secretions in hypophysectomized male rats and resulted in an increase in CYP2C11 mRNA expression, as well as 2α-OHT activity (Waxman et al., 1991). Although twice daily subcutaneous injections of GH is known to increase CYP2C11 to normal levels in hypophysectomized rats, we show that administration of GH in the same fashion to intact rats causes a decrease in CYP2C11. GH administration (with CsA vehicle) significantly reduced CYP2C11 expression when compared with the corresponding GH vehicle group. Nearly the same result has also been obtained by other researchers using the same route of administration, dose, and a comparable length study period of 12 days (Kawai et al., 2001). This could indicate that exogenous GH, in addition to endogenously secreted GH, is causing the animals to continually have GH present in circulation, effectively increasing the basal level of GH. The immediate downstream consequence of the substantially higher concentration of GH in plasma may be that the GH receptor is not functioning in the correct capacity to initiate the JAK/STAT

pathway. This notion is supported by the GH receptor dimerization theory, whereby GH receptors only function to trigger the intracellular signal transduction necessary for initiation of P450 protein gene transcription when they are bound to one GH molecule and then dimerize with another GH receptor molecule (Fuh et al., 1992). Therefore, excess GH in circulation may lead to a 1:1 ratio of binding and would not allow for the 1:2 ratio of GH:GH receptor necessary for initiation of action by GH.

A significant decrease in the production of 2α-OHT and 16α-OHT was exhibited with the administration of CsA alone as compared to the administration of CsA vehicle alone. This is consistent with previous results showing a suppression of CYP2C11 protein expression and enzyme activity as a result of chronic CsA administration (Brunner et al., 1996b). The administration of CsA in combination with GH resulted in a further suppression of CYP2C11 activity than when either agent was administered with vehicle. Although this is in slight discordance with protein levels, the trend appears similar although the results were not statistically significant for the protein expression data. Considering that CYP2C11 is the predominant P450 enzyme present in male rat liver, the additive suppressive effect of combined GH and CsA therapy on CYP2C11 activity may have profound consequences on drug metabolism overall.

GH secretion profile parameters were not significantly altered due to CsA treatment, which revealed that chronic CsA treatment does not alter the level of GH in circulation. Since modifying the levels of GH can lead to a change in CYP3A2 and CYP2C11 levels, CsA does not seem to be affecting P450 expression by modulating GH levels. GH may still mediate the suppression of P450 enzymes by CsA at the receptor-binding or signal transduction level. Conversely, GH did not have an effect on detectable CsA concentrations in blood either. Because the combined administration of CsA and GH produced significantly lower CYP3A2 activity than when CsA was given with vehicle, we expected to see a considerably higher concentration of CsA in blood as a result of decreased CsA metabolism by CYP3A2. However, only a slight elevation was observed. This may be due to CsA being sequestered into organs, including the liver, kidney, and small intestine, thus preventing detection in blood.

The data also suggest that changes in hormonal status is likely to be one of the many factors that is responsible for the lack of a clear association between cyclosporine dosing and markers of toxicity. Hormonal status can be altered by either manipulating the amount of hormone in circulation or the subsequent signaling action of the hormone. Modulation of GH action can occur by one or more of several possible methods including a modification of the binding capacity of circulating GH, alteration of GH receptor (GHR)

quantity or binding capability and/or availability, alteration of Janus kinase-2 (JAK-2) action, or modification of any one of the GH-activated intracellular signaling pathways (Frank et al., 2000). According to this rationale, an alteration anywhere along the GH activation pathway could affect intracellular signaling and eventually, the downstream gene regulation of P450 protein production. Future studies are warranted to investigate GH binding and the signal transduction mechanism behind the possible function of GH in the alteration of P450 expression and activity by CsA.

Table 2.1 Renal function parameters

Parameter	CsA	CsA V	GH/CsA	GH/CsA V	GH V/CsA	GH V/CsA V
Weight Change (%)	5.6 ± 2.3*	10.8 ± 1.6	7.4 ± 1.8*	19.7 ± 0.6	5.7 ± 1.9*	17.5 ± 1.1
Urine Volume (ml)	17.5 ± 2.0*	9.0 ± 2.1	11.0 ± 1.4†	12.2 ± 2.8	18.3 ± 3.8*	7.8 ± 0.6
Scr (mg/dl)	0.66 ± 0.04	0.60 ± 0.03	0.76 ± 0.07*	0.58 ± 0.06	0.67 ± 0.06	0.70 ± 0.07
Clcr (µl/min/100 g)	283 ± 53	391 ± 37	268 ± 29	340 ± 28	418 ± 97	309 ± 28

Table 2.1 * = P<0.05 as compared to the respective CsA vehicle control group.
† = P<0.05 as compared to the respective GH vehicle control group

Table 2.2 GH release profile parameters

Parameter	CsA	CsA V	GH/CsA	GH/CsA V	GH V/CsA	GH V/CsA V
Mean Peak Amplitude	83.4 ± 35.5	90.6 ± 35.1	173.0 ± 38.1	152.3 ± 17.1	127.3 ± 30.4	178.9 ± 30.5
Mean Peak Number	4.8 ± 0.6	5.0 ± 0.4	5.4 ± 0.5	5.0 ± 0	5.7 ± 0.5	5.7 ± 0.6
Mean Peak Duration	71.0 ± 8.1	64.3 ± 2.9	58.4 ± 3.6	66.8 ± 3.1	51.7 ± 2.2	63.7 ± 9.8

Table 2.2 All units are ng GH/ml of serum. No statistically significant differences were found between drug treatment groups and respective vehicle groups.

Figure 2.1 Effects of CsA and GH treatment on CYP3A1/2 and CYP2C11 expression

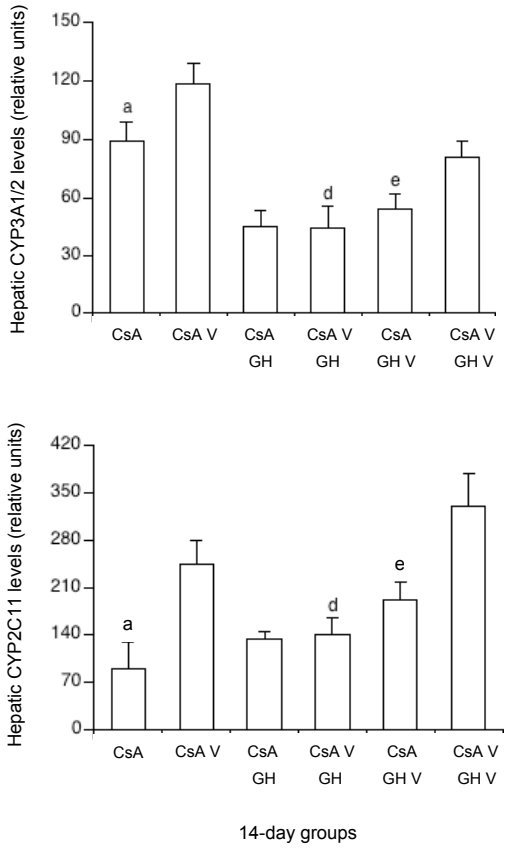


Figure 2.1 Western blot analysis of hepatic CYP3A1/2 and CYP2C11 microsomal protein expression in 14-day groups. Groups were given subcutaneous doses of either 15 mg/kg CsA or CsA vehicle alone or a combination of GH, GH vehicle, CsA, and CsA vehicle. All values are expressed as a percent density of the protein standard used for each blot. **a**=$p<0.05$ between C and CV groups, **d**=$p<0.05$ between G/CV and GV/CV groups, **e**=$p<0.05$ between GV/C and GV/CV groups.

Figure 2.2 Effects of CsA and GH treatment on CYP3A1/2 and CYP2C11 activity

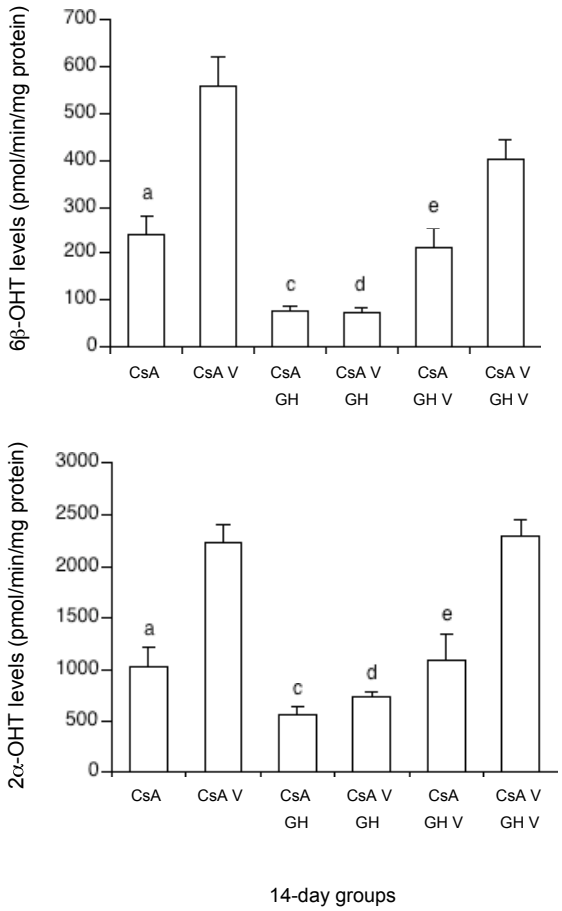


Figure 2.2 *In vitro* testosterone hydroxylation by hepatic microsomes following 14 days of treatment. Top graph represents 6β-hydroxylase activity; bottom graph represents 2α-hydroxylase activity. Units for metabolite production are pmol metabolite/min/mg microsomal protein. a=$p<0.05$ between C and CV groups; c=$p<0.05$ between G/C and GV/C groups; d=$p<0.05$ between G/CV and GV/CV groups; e=$p<0.05$ between GV/C and GV/CV groups.

Figure 2.3 AUC of circulating GH levels

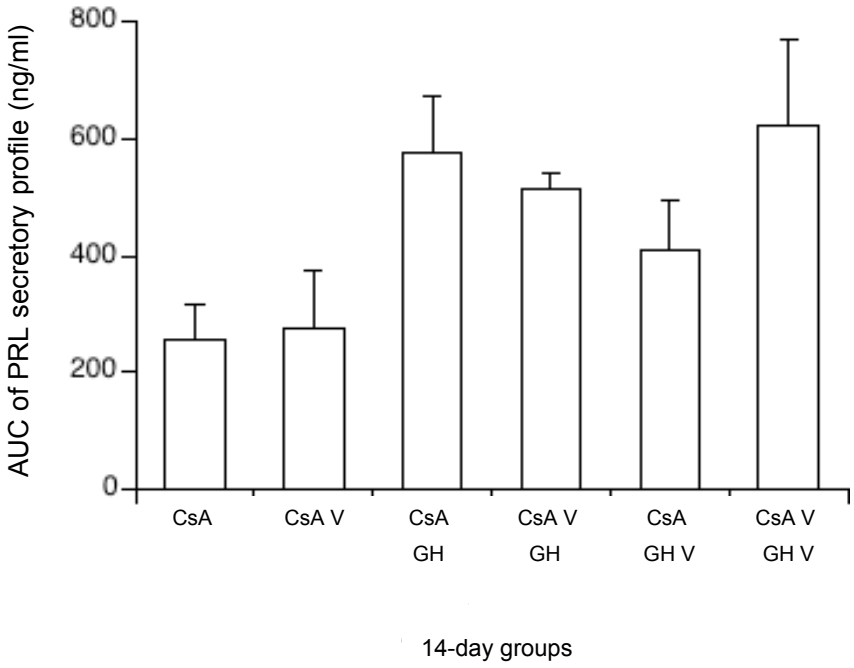


Figure 2.3 Area under the curve calculations of GH secretory profiles for circulating serum GH. Units are expressed as ng of circulating GH per ml of serum.

Figure 2.4 Representative graphs of GH secretory profiles

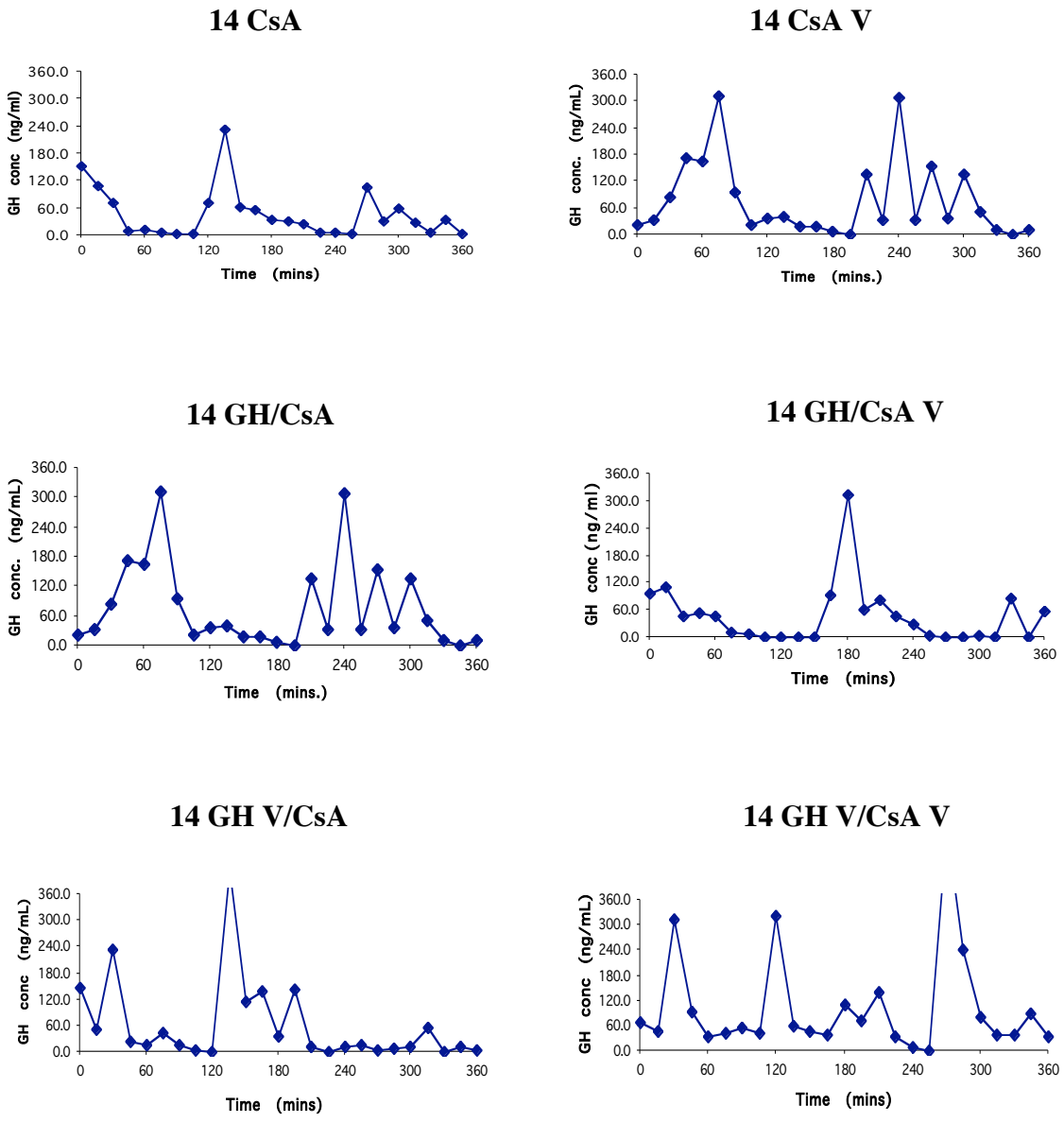


Figure 2.5 Western blot densitometry

Liver 3A1/2 representative blot

Liver 2C11 representative blot

Figure 2.5 Representative blots for liver 3A1/2 and 2C11 protein expression, respectively. One representative animal was selected from each group for the densitometry figure. From left to right, the groups are: 14C, 14CV, 14G/C, 14G/CV, 14GV/C, 14GV/CV.

CHAPTER THREE

Cyclosporine and Bromocriptine-Induced Suppressions of CYP3A1/2 and CYP2C11 are Not Mediated by Prolactin

3.1 INTRODUCTION

A connection between the neuroendocrine and the immune system has been shown to exist in various ways. Prolactin (PRL), a peptide hormone produced in the pituitary, is part of a superfamily of hormones that includes growth hormone and placental lactogens. Although the chief functions of PRL are maintaining lactation and stimulating mammary gland growth, this lactogenic hormone has been demonstrated to be one of the critical components in maintaining immune function (Gala, 1991; Nagy and Berczi, 1978). This role in the immune system ranges from driving T-cell proliferation in murine T-cell clone L2 cells (Clevenger et al., 1990) to inducing interleukin-2 (IL-2) receptors on ovariectomized female rat splenic lymphocytes (Mukherjee et al., 1990).

Alterations in the level of circulating PRL can cause immune dysfunction. When male rats were treated with ovine PRL, their ability to reject parasitic infection was suppressed (Kelly and Dineen, 1973). Hypoprolactinemia, can also result in a failed immune response. This condition can be artificially produced in animals by the administration of bromocriptine (BRC) (Nagy et al., 1983). BRC is a dopamine receptor agonist that inhibits PRL secretion. This ergopeptide is commonly used in the treatment of hyperprolactinemia, Parkinsonism, and acromegaly (Vance et al., 1984). BRC's effect on the endocrine system has been clearly demonstrated, however specific studies on the effect of BRC on hepatic drug metabolism have been limited.

Cyclosporine (CsA) is a widely used immunosuppressant drug given to patients following organ transplantation. CsA is an undecapeptide that blocks transcription of genes for IL-2 at the mRNA level, thus inhibiting helper T-cell proliferation (Granelli-Piperno, 1988; Kronke et al., 1984). Although it has been well established that the relationship between CsA and PRL is antagonistic, the exact mechanism of how this antagonism takes place is not certain. At specified concentrations, CsA has been shown to completely inhibit PRL binding to lymphocytes of human mononuclear cells (Russell et al., 1985). This concept was challenged by the finding that CsA inhibited the prolactin-stimulated growth of the rat lymphoma Nb-2 cell line without affecting PRL binding to receptors (Varma and Ebner, 1988).

Within the liver, CsA is almost exclusively metabolized by the CYP3A isoform family of the cytochrome P450 (P450) system in humans and animals (Kronbach et al., 1988; Maurer, 1985), although other isoforms may also be involved (Brunner et al., 1996a; Prueksaritanont et al., 1993a). Therefore, factors that affect the P450 system should also be suspected of affecting the metabolism of CsA. For example, when hepatic P450 levels were increased in rats, the nephrotoxic effects of CsA were lessened (Cunningham et al., 1985). While CsA is metabolized by P450 enzymes, CsA also has an effect on the disposition of these enzymes. Chronic treatment with moderate daily doses of CsA suppresses protein levels and catalytic activity of the CYP3A2 and CYP2C11 isoforms in the rat liver (Brunner et al., 1996a). Furthermore, this inhibition occurs in a time-dependent (Brunner et al., 1998a) and dose-dependent (Brunner et al., 2000) manner. Because CsA is not only a substrate, but also an inhibitor of CYP3A, chronic CsA therapy can result in a cycle of protein suppression and accumulation of drug in the body that eventually leads to organ toxicity.

Few studies have examined the possible interrelationship between PRL, immune suppression, and the effects on hepatic drug metabolism. The aim of our study was two-fold: first, to determine if the suppression of hepatic CYP3A and CYP2C11 by CsA is mediated by PRL and second, to investigate the effects of BRC on hepatic drug metabolism. The results from this study will aid

in the understanding of how hormonal status can affect drug metabolism in the body and attempt to untangle the complex relationship between the endocrine system, immune function, and drug metabolism.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Unless otherwise noted, all chemicals were purchased in the highest purity available from EM Science (Gibbstown, NJ, USA). Cyclosporine was generously provided by Novartis (East Hanover, NJ, USA) in the form of Sandimmune™ oral solution. The original dosage form was diluted in commercially available olive oil and stored in amber bottles to limit light exposure. The cyclosporine vehicle was the same commercially available olive oil. Bromocriptine was purchased in the form of 2-bromo-α-ergocryptine methanesulfonate salt (Sigma-Aldrich, St. Louis, MO, USA) and dissolved in 70% ethanol along with an equivalent weight of tartaric acid. This solution was then suspended in pure olive oil at a concentration of 4.6 mg/ml of bromocriptine with the percentage of ethanol not exceeding 12.5% of the total volume of the suspension. Purified ovine prolactin was purchased from the National Hormone and Pituitary Program under the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK, Torrance, CA, USA) and

solubilized in 0.03M $NaHCO_3$ in 0.15M NaCl at a final pH of 8.5. Prolactin vehicle consisted of 0.03M $NaHCO_3$ in 0.15M NaCl. Ovine prolactin has been shown to have similar physiological function to rat prolactin when examined for its effectiveness in eliciting tyrosine kinase activity in Nb2 cells (Rillema et al., 1992) and restoration of lactation in bromocriptine-treated female rats (Hebert et al., 1993).

The anesthetic mixture consisted of a 1:1:1 (v:v:v) ratio of ketamine (100 mg/ml), xylazine (20 mg/ml), and acepromazine (10 mg/ml). Ketamine and xylazine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acepromazine was purchased from Fort Dodge Laboratories, Inc. (Fort Dodge, LA, USA). Rat CYP3A1/2 and CYP2C11 selective polyclonal antibodies were purchased from Gentest Corporation (Woburn, MA, USA). Rat CYP3A1/2 antibody was isolated from goats that were immunized with CYP3A2 purified from rat liver. Due to the polyclonal nature of the antiserum, two indistinguishable bands (CYP3A1 and CYP3A2) are detected and are therefore collectively referred to as CYP3A1/2. The microsomal standard used for the relative quantitation of CYP3A1/2 protein was composed of phenobarbital-treated rat liver microsomes (Xenotech, Lenexa, KS, USA). Rat CYP2C11 antibody is also polyclonal and raised in goats, however the cross reactivity with CYP2C13 can be readily distinguished from CYP2C11 based on mobility. CYP2C11 standard was composed of dexamethasone-treated male rat liver

microsomes (Xenotech, Lenexa, KS, USA). The horseradish peroxidase-conjugated rabbit anti-goat (secondary P450 antibody) was purchased from ICN Pharmaceuticals, Inc. (Aurora, OH, USA). Rat prolactin enzyme immunoassay (EIA) kits were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA) and were manufactured by Spi-Bio (Massy Cedex, France).

3.2.2 Animals

All procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas at Austin and were in accordance with the guidelines established by the National Institutes of Health for the humane treatment of animals. Eight- to nine-week old, male Sprague-Dawley rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). During the study, all rats were kept in a 12-h light/dark cycle environment with free access to low-salt rat chow (Harlan, Indianapolis, IN, USA) and deionized water. Due to the rat's resistance to nephrotoxicity during cyclosporine therapy, low-salt rat chow was given to predispose the animals to develop chronic cyclosporine nephropathy similar to that in humans (Shihab et al., 1997). Food consumption and body weight were measured daily.

Rats were randomly assigned to one of eight groups (N=6). The groups are as follows: +PRL/+CsA, +PRL/-CsA, -PRL/+CsA, -PRL/-CsA, +BRC/+CsA, +BRC/-CsA, -BRC/+CsA, and -BRC/-CsA (PRL = prolactin, CsA = cyclosporine, BRC = bromocriptine). Animals receiving prolactin were

administered twice daily subcutaneous doses of 500 ng/g body weight of ovine prolactin. The prolactin vehicle was administered in the same fashion as prolactin at 1 ml/kg. Cyclosporine administration consisted of once daily subcutaneous injections of a 15 mg/kg dose and cyclosporine vehicle was also administered once daily at a dose of 1 ml/kg.

3.3.3 Blood collection

Two days prior to blood collection, jugular cannulas were implanted surgically in all animals as previously described (Waynforth and Flecknell, 1992). Twenty-four hours prior to blood collection, rats were placed into standard rodent metabolic cages for passive urine collection. Following this 24-h period, 0.1 ml blood samples were collected once every 15 minutes for four hours via the indwelling jugular cannula. Plasma volume was replaced with sterile saline. After collection, the blood was allowed to clot on ice and was centrifuged at 9000 x *g* for 5 minutes at 4° C. Serum was harvested immediately and stored at –80° C until assayed.

3.3.4 Microsome isolation

Upon sacrifice of the animals, livers were excised immediately. Liver microsomes were isolated with modifications of a previously described method of differential centrifugation (Coon et al., 1978b) and kept at 4° C during the entire preparation. Briefly, liver tissue was ground in three volumes of Tris

chloride buffer consisting of 1 mM EDTA and 0.15 M potassium chloride using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA, USA). Samples were then centrifuged and resuspended in a Tris chloride buffer containing 1 mM EDTA and 0.2 M sodium pyrophosphate. After the final spin, the supernatant was discarded and the pellet washed and homogenized in Tris buffer containing 20% glycerol for storage. The microsomes were then stored at -80°C until analysis.

3.3.5 Gel electrophoresis and immunoblot analysis

SDS-PAGE (sodium docecyl sulfate-polyacrylamide gel electrophoresis) was performed as previously described (Laemmli, 1970) using an 8% polyacrylamide separating gel. Twenty-five micrograms of protein was loaded onto the gel and transferred to nitrocellulose sheets by a prior described method (Schnier et al., 1989b). After protein transfer, the nitrocellulose sheets were blocked with 3% non-fat dry milk in Tris-buffered saline (TBS) at room temperature. Detection of putative proteins was achieved with goat anti-rat IgG (in a 1:2000 dilution) that was immunoreactive to the specific P450 enzyme of interest in 3% non-fat dry milk and then rabbit anti-goat horseradish peroxidase (in a 1:2000 dilution) also in 3% non-fat dry milk at room temperature. Immune complexes for CYP3A1/2 and CYP2C11 were detected with an NEN chemilumnescence reagent kit as described by the manufacturer (New England Nuclear Life Science Products, Boston, MA, USA). Blot densities were

measured using a flatbed scanner (Microtek, Hsinchu, Taiwan) and analyzed on a Dell PC computer using the Kodak 1D image analysis software, version 3.5 (Eastman Kodak Co., Rochester, NY, USA).

3.3.6 Testosterone/BRC Hydroxylation Assay and HPLC assay

Liver samples for the testosterone hydroxylation assay were prepared as previously described (Brunner et al., 1996a). In brief, 200 μg of liver microsomal protein was added to 0.02M potassium phophate buffer (pH 7.4), regeneration system, and water up to 1 ml. The samples were then incubated with 250 μM testosterone for 3 minutes at 37° C followed by the addition of glucose-6-phosphate dehydrogenase (1 unit/μl) for 15 minutes under the same conditions. The reaction was quenched with dichloromethane and 11α-hydroxyprogesterone (1.2 μg) was added as the internal standard. The organic phase was transferred and evaporated, then dissolved in methanol and stored at 4° C. The testosterone/bromocriptine inhibition assay was carried out in similar fashion to the testosterone hydroxylation assay with the exception that bromocriptine was added at a concentration of 107 μM to each tube at the same time as the testosterone. The volume of bromocriptine was accounted for by subtracting an equal volume from the water component in each sample tube.

Separation and detection of testosterone and metabolites were performed on a Shimadzu high-performance liquid chromatography (HPLC)

system (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). The column, a Supelcosil LC-18 (Sigma-Aldrich, St. Louis, MO, USA), was kept at a constant temperature of 40° C and a wavelength of 238 nm was used to detect the analytes. All other conditions were identical to our previously described method (Brunner et al., 2000). Peak areas of corresponding hydroxylation metabolites were measured and compared to peak areas of the internal standard within the same run.

3.3.7 rPRL Enzyme Immunoassay

The enzyme immunoassay used to determine prolactin levels was manufactured by Spi-Bio (Massy Cedex, France). The principle of the assay is based on the competition between unlabelled rat prolactin and acetylcholinesterase bound to a tracer. The yellow color formed by exposing the sample and acetylcholinesterase to an enzymatic substrate (and chromagen) for acetylcholinesterase was measured spectrophotometrically. The limit of detection for the assay was 0.5 ng/ml.

3.3.8 Data Analysis

Sample densities from Western blots were compared with standard microsomal protein densities and expressed as a percentage. Prolactin area under the curve (AUC) values represent the total area under the entire secretion profile curve of each animal and then averaged for all values within each

treatment group. One-way ANOVA (analysis of variance) and *a priori* means comparisons tests were employed using respective vehicle groups as the control with the aid of the SuperANOVA statistical program (Abacus Concepts, Inc., Berkeley, CA, USA). Data are presented as mean ± standard error. When the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$), the differences were then considered to be statistically significant.

3.3 RESULTS

3.3.1 Effect of CsA and alterations in PRL hormonal status on hepatic P450 protein levels

Cyclosporine administered over a 14-day period has been shown to significantly reduce CYP3A1/2 protein expression in the male rat liver (Brunner et al., 1998a). Fig. 3.1 shows that cyclosporine given in the presence of either prolactin or prolactin vehicle resulted in a decrease in CYP3A1/2 expression ($P<0.01$; $P=0.063$, respectively) as compared with cyclosporine vehicle control groups. The administration of prolactin did not cause a notable difference in the protein levels of CYP3A1/2, either when given in combination with cyclosporine or cyclosporine vehicle. Conversely, when cyclosporine was administered concomitantly with either bromocriptine or bromocriptine vehicle, there was a significant decline in both cases ($P<0.01$; $P<0.001$, respectively). In

addition, the introduction of bromocriptine, which effectively abolishes prolactin secretion, resulted in a significant decrease in the expression of CYP3A1/2 in the absence of cyclosporine ($P<0.001$). There was no difference in suppression when cyclosporine was present along with bromocriptine as compared with the bromocriptine vehicle/cyclosporine group ($P=0.205$), indicating the lack of an additive suppression of CYP3A1/2 with the administration of both bromocriptine and cyclosporine. However, the addition of cyclosporine did further contribute to the existing suppression caused by bromocriptine and cyclosporine vehicle ($P<0.001$).

Suppression of CYP2C11 by chronic cyclosporine treatment has been documented before (Brunner et al., 1998a) and is confirmed by our present study. However, administration of exogenous prolactin did not have a significant effect on CYP2C11 protein expression (Fig. 3.2). Conversely, bromocriptine and cyclosporine separately caused a significant decrease in CYP2C11 ($P<0.05$; $P<0.05$, respectively) and the combination of the two drugs caused even greater decreases in protein expression when compared to the +BRC/-CsA and -BRC/+CsA groups ($P<0.01$; $P<0.01$, respectively).

3.3.2 Effect of CsA and alterations in PRL hormonal status on hepatic P450 activity levels

The *in vitro* conversion of testosterone to 6β-hydroxytesterone (6β-OHT) is primarily catalyzed by CYP3A2 (Waxman et al., 1985; Waxman et al.,

1983) and to a lesser extent by CYP3A1 (Sonderfan et al., 1987). Thus, the measure of 6β-OHT formation is a reliable indicator of CYP3A1/2 activity levels. A testosterone hydroxylation assay and subsequent HPLC analysis revealed that cyclosporine significantly impaired the formation of 6β-OHT in the liver when given in combination with prolactin vehicle ($P<0.05$) or with prolactin ($P<0.05$) (Fig. 3.3). The administration of prolactin did not change 6β-OHT levels, confirming the CYP3A1/2 protein measurements. However, when prolactin secretion is depleted with bromocriptine treatment, CYP3A1/2 activity was significantly lowered. This was evident in the presence ($P<0.01$) and in the absence of cyclosporine ($P<0.001$).

The formation of 2α-OHT mainly corresponds to the catalytic activity of CYP2C11 (Waxman, 1984a). Cyclosporine significantly lowered CYP2C11 activity by 27% both when given in combination with prolactin or prolactin vehicle (Fig. 3.3). However, the effect of excess exogenous prolactin alone did not produce a significant change in CYP2C11 activity. Bromocriptine caused a decrease in CYP2C11 activity in the absence of cyclosporine, similar to protein expression results. Upon bromocriptine administration with cyclosporine, 2α-OHT levels were significantly lower than from the effects of bromocriptine or cyclosporine alone ($P<0.001$; $P<0.001$, respectively).

3.3.3 *In vitro* effect of BRC on CYP3A- and CYP2C11-catalyzed testosterone hydroxylation

The observation that bromocriptine in combination with cyclosporine additively decreased CYP3A1/2 and CYP2C11 activity led us to investigate if there was a direct competition between bromocriptine and testosterone for enzyme activity *in vitro* causing decreased *in vitro* testosterone metabolite production. We found that the presence of bromocriptine suppressed the production of 6β-OHT by 35% ($P<0.05$). However, the presence of bromocriptine did not significantly alter the production of 2α-OHT *in vitro* ($P=0.33$) (Fig. 3.6).

3.3.4 Circulating endogenous serum prolactin levels

Because prolactin secretion in the rat has been described as episodic in nature (Lafuente et al., 1996), collection of blood samples took place over a 4-h period. The serum from these samples was analyzed for prolactin and a graph of concentration versus time was generated for each animal. These graphs were used to produce area under the curve (AUC) calculations and the values were averaged within each group (Fig. 3.4).

Cyclosporine had a significant inductive effect on circulating prolactin levels. As shown in a previous study (Lafuente et al., 1996), chronic cyclosporine treatment resulted in an induction of rat prolactin levels. In

addition, our study shows a 104% increase in AUC of circulating prolactin levels ($P<0.001$) caused by long-term cyclosporine therapy. However, when compared with cyclosporine vehicle, the introduction of cyclosporine had virtually no effect when bromocriptine was co-administered ($P=0.841$). Conversely, bromocriptine was able to significantly lower prolactin levels even in the presence of the inductive effects of cyclosporine ($P<0.001$) when compared with the administration of bromocriptine vehicle and cyclosporine. As anticipated, bromocriptine administration resulted in a decrease in endogenous circulating prolactin levels as compared with the administration of bromocriptine vehicle ($P<0.05$) as shown in representative graphs in Fig. 3.4. The administration of prolactin over 14 days failed to cause an increase in rat prolactin AUC levels. This is in accordance with previous studies showing little effect of the administration of ovine prolactin on the endogenous secretion of prolactin in ovariectomized female rats (Luquita et al., 1996).

3.3.5 Effect of BRC alone and in combination with PRL

In order to examine if the effects of bromocriptine on CYPs were a direct result of depleting prolactin levels or another mechanism, we also investigated the effect of administering both bromocriptine and prolactin concomitantly. This portion of the study entailed treating two groups of animals with either bromocriptine in combination with prolactin or bromocriptine in combination with prolactin vehicle. Re-introducing prolactin to animals treated

with bromocriptine served to eliminate the effect of prolactin as the cause of altered drug metabolism. In other words, the results of this experiment would help clarify if bromocriptine is truly suppressing hepatic CYPs through the reduction of prolactin or if the mechanism is independent of its effects on prolactin disposition. When bromocriptine and prolactin are given together, the hormonal status of the animal theoretically mimics a normal *in vivo* situation. Likewise, when bromocriptine is given with prolactin vehicle, the resultant effect is a normal animal with lowered prolactin levels due to the bromocriptine.

CYP3A1/2 protein levels were slightly lower for animals treated with bromocriptine/prolactin as compared with animals treated with vehicle (Fig. 3.5). However, the difference was not statistically significant. The production of 6β-OHT remained unchanged between the groups (Fig. 3.5). Similarly, CYP2C11 protein expression and activity were not significantly modified.

3.4 DISCUSSION

We have shown previously that chronic cyclosporine dosing results in a demasculinization of hepatic P450 protein expression in rats (Brunner et al., 1996a; Brunner et al., 1998a). This change in P450 expression was postulated to be due to an effect on growth hormone secretion. More recently, we have

demonstrated that cyclosporine does not modulate these P450 proteins by altering circulating growth hormone levels (Lu et al., 2003b). This does, however, leave the possibility that another pituitary hormone such as prolactin may play a role in the suppressive effect of cyclosporine. Due to its role in the immune system and the ability to act locally upon its release, prolactin has been rightfully viewed as a cytokine for roughly the past decade. With prolactin's immunomodulatory function in mind, we investigated this hormone's role in the suppressive action of cyclosporine on hepatic P450 enzymes. This concept was examined by independently supplementing and abolishing prolactin release in an *in vivo* system and analyzing the resultant effect on P450 enzymes while in the presence or absence of chronic cyclosporine therapy.

The CYP3A family of P450 enzymes contributes significantly to the overall drug metabolism that takes place in the rat liver. Since cyclosporine is both a substrate and an inhibitor of CYP3A2, this isoform was of primary importance for this study. Results from the CYP3A1/2 protein expression data suggest that drug metabolism is influenced more by the absence of prolactin secretion rather than the super-physiological state of the hormone (Fig. 3.1). Previous studies involving treatment with prolactin did not directly address the effect of excess prolactin on the disposition of CYP3A1/2. We show that chronic subcutaneous administration of prolactin had no effect on CYP3A1/2

protein levels or activity. Also, prolactin did not alter the suppressive effect of cyclosporine on this enzyme.

While prolactin supplementation did not significantly alter the expression of CYP3A1/2, to fully understand the overall maintenance of hepatic drug metabolism, the effect of abolishing prolactin also needed to be investigated. When bromocriptine (an inhibitor of prolactin secretion) was administered in chronic subcutaneous doses, it not only resulted in a suppression of CYP3A1/2 protein expression and activity, but it also augmented the suppression in 6β-OHT production caused by cyclosporine. To investigate the possible cause of this additive suppression, we incubated hepatic microsomal samples from a vehicle-treated animal in the presence of both bromocriptine and testosterone as metabolic substrates. The results showed that the presence of bromocriptine at a concentration of less than one half of testosterone decreased the amount of CYP3A-catalyzed testosterone hydroxylation product, but had no significant effect on CYP2C11 activity. This result indicates that bromocriptine is competing with testosterone for P450 metabolism, thus lending less enzyme activity towards testosterone metabolism. Since CYP3A has been shown to oxidize bromocriptine in rat liver microsomes (Peyronneau et al., 1994b), bromocriptine is likely suppressing the production of 6β-OHT through competition with testosterone for CYP3A-catalyzed metabolism. Based on this information, bromocriptine could also compete with

cyclosporine for CYP3A metabolism, leading to an accumulation of cyclosporine and resulting in a further decrease in overall CYP3A expression and activity.

In order to confirm bromocriptine's effects are due to the alteration of prolactin, in two separate groups, we supplemented prolactin during bromocriptine treatment and compared those effects to a prolactin vehicle group. Our data demonstrate that supplemental doses of prolactin did not overcome the suppressive effect of bromocriptine on CYP3A1/2 or CYP2C11. This suggests that prolactin is not the mediating factor in the suppressive effect of bromocriptine. However, as only one concentration of bromocriptine and prolactin were used, it remains a possibility that the prolactin dose was not sufficient enough to overcome the effects of the sizeable dosage of bromocriptine.

The expression of CYP2C11 is obligatorily dependent on the pulsatile secretions of growth hormone in the adult male rat (Waxman et al., 1991). Without the presence of differentiable pulses and trough periods, hepatic CYP2C11 will fail to express. Since CYP2C11 is highly dependent on the pattern of growth hormone secretion, this hormonal dependence may extend to other hormones with gender-dependent secretion, such as prolactin. The reduction of prolactin secretion by bromocriptine suppressed CYP2C11 protein expression (Fig. 3.2). This effect was also observed in CYP2C11 activity from

bromocriptine-treated rats (Fig. 3.3). As with the CYP3A1/2 data, the depletion of prolactin is more dominant in affecting CYP2C11 activity than the presence of excess prolactin. Additionally, the data from the *in vitro* experiments revealed that the levels of CYP2C11-catalyzed product did not change in the presence of bromocriptine. This provides evidence that the *in vivo* suppression of CYP2C11 activity by bromocriptine is not an artifact of competition with bromocriptine for metabolism, as is the case for CYP3A1/2. Thus, the effect on CYP2C11 initiated by bromocriptine is likely due to actual suppressive actions of bromocriptine. Although reports of the role of prolactin in drug metabolism are limited in the literature, one study did demonstrate that there were no differences in levels of CYP2C11 in hypophysectomized female rats treated with prolactin as compared with untreated hypophysectomized rats (Yamazoe et al., 1987). While the replacement of prolactin cannot induce the production of male-specific P450 enzymes in the female rat, we have shown that the lack of prolactin can decrease CYP2C11 in the male rat, demonstrating the importance of maintaining prolactin status *in vivo*. Given that the expression of CYP2C11 is strongly dependent on the levels of circulating growth hormone, it is reasonable that CYP2C11 is also particularly responsive to levels of circulating prolactin.

As anticipated, bromocriptine effectively depressed circulating prolactin levels as evidenced by the AUC of prolactin concentrations in blood collected

over time (Fig. 3.4). Circulating prolactin levels increased with the administration of cyclosporine but not when bromocriptine was given at the same time. This indicates that prolactin inhibition caused by bromocriptine dominates over the induction of prolactin secretion by cyclosporine. Others have also reported that chronic cyclosporine dosing significantly increased the circulating levels of prolactin in rats (Lafuente et al., 1996). However, the data from that particular study showed that when using an ectopic pituitary graft in conjunction with cyclosporine treatment, the levels of prolactin are lower than with the ectopic graft alone. The use of an ectopic pituitary graft often complicates the investigation of hormonal effects since the pituitary releases a host of other hormones such as growth hormone, which is known to alter CYP3A2 and CYP2C11 expression. For this reason, we chose to selectively supplement prolactin using twice daily injections, so as to eliminate potential interferences from other pituitary hormones. Our study indicates that chronic cyclosporine treatment induces circulating prolactin levels by promoting endogenous secretion of prolactin.

Although growth hormone plays a key role in the male-specific expression of CYP's, the secretion of prolactin does not seem to be as critical in the proper maintenance of CYP3A2 and CYP2C11expression. Accordingly, cyclosporine-induced suppression of CYP3A1/2 and CYP2C11 is likely not mediated by prolactin. We have also shown that while bromocriptine

suppresses CYP3A1/2 and CYP2C11, it is likely acting via a different mechanism than inhibiting prolactin release. This drug may have intrinsic suppressive action and may be of considerable clinical significance to further investigate bromocriptine's effects on hepatic drug metabolism. Overall, cyclosporine and bromocriptine both appear to affect drug metabolism through prolactin-independent suppression of CYP3A and could affect human drug metabolism in a similar fashion as well.

Figure 3.1 Effects of cyclosporine, prolactin, and bromocriptine on CYP3A1/2 protein expression

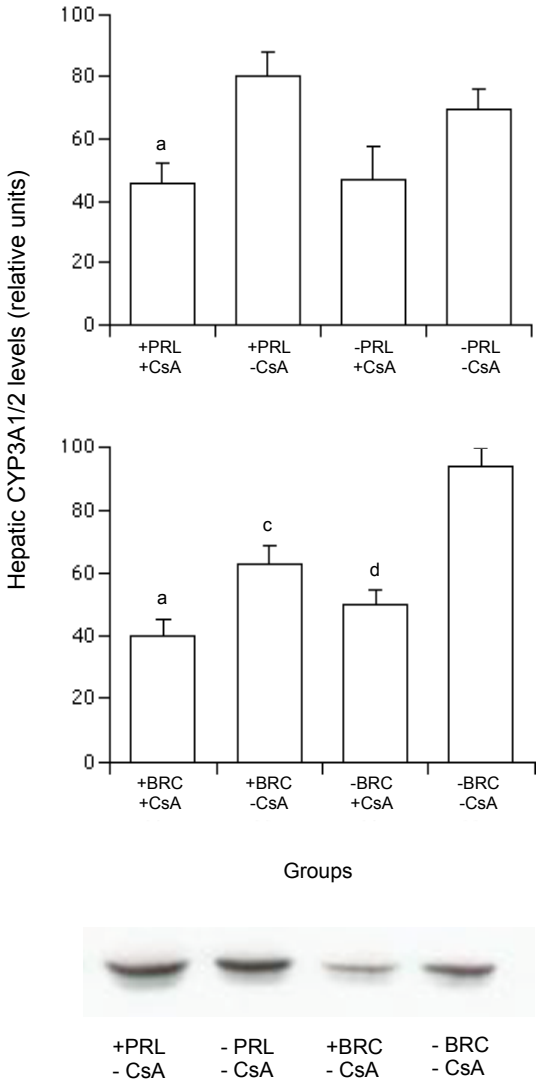


Figure 3.1

Western blot analysis of hepatic CYP3A1/2 microsomal protein expression in 14-day groups. Groups were given subcutaneous doses of either 15 mg/kg cyclosporine (+CsA) or cyclosporine vehicle (–CsA) in combination with

prolactin (+PRL), prolactin vehicle (−PRL), bromocriptine (+BRC), or bromocriptine vehicle (−BRC). All values are expressed as a percent density of the protein standard used for each blot. **a**=P<0.05 between +PRL/+CsA and +PRL/−CsA (top graph) or between +BRC/+CsA and +BRC/−CsA groups (bottom graph), **c**=P<0.05 between +BRC/−CsA and −BRC/−CsA groups, **d**=P<0.05 between −PRL/+CsA and −PRL/−CsA (top graph) or between −BRC/+CsA and −BRC/−CsA groups (bottom graph).

Figure 3.2 Effects of cyclosporine, prolactin, and bromocriptine on CYP2C11 protein expression

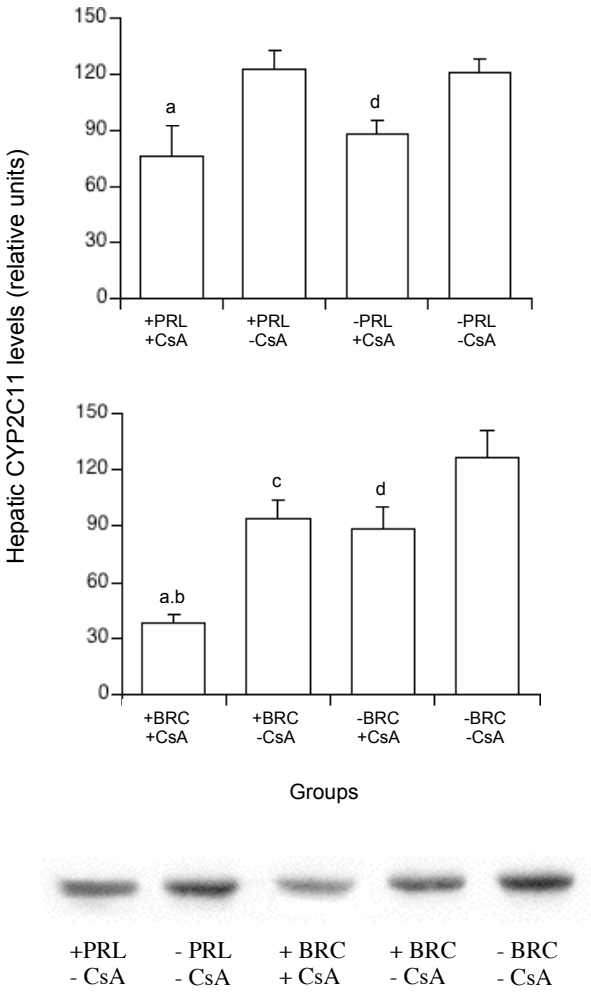


Figure 3.2

Western blot analysis of hepatic CYP2C11 microsomal protein expression in 14-day groups. Groups were given subcutaneous doses of either 15 mg/kg cyclosporine or cyclosporine vehicle in combination with prolactin, prolactin vehicle, bromocriptine, or bromocriptine vehicle. All values are expressed as a

percent density of the protein standard used for each blot. a=P<0.05 between +PRL/+CsA and +PRL/−CsA (top graph) or between +BRC/+CsA and +BRC/−CsA groups (bottom graph), b=P<0.05 between +PRL/+CsA and −PRL/+CsA (top graph) or between +BRC/+CsA and −BRC/+CsA groups (bottom graph), c=P<0.05 between +BRC/−CsA and −BRC/−CsA groups, d=P<0.05 between −PRL/+CsA and −PRL/−CsA (top graph) or between −BRC/+CsA and −BRC/−CsA groups (bottom graph).

Figure 3.3 Effects of cyclosporine, prolactin, and bromocriptine on CYP3A1/2 and CYP2C11 activity

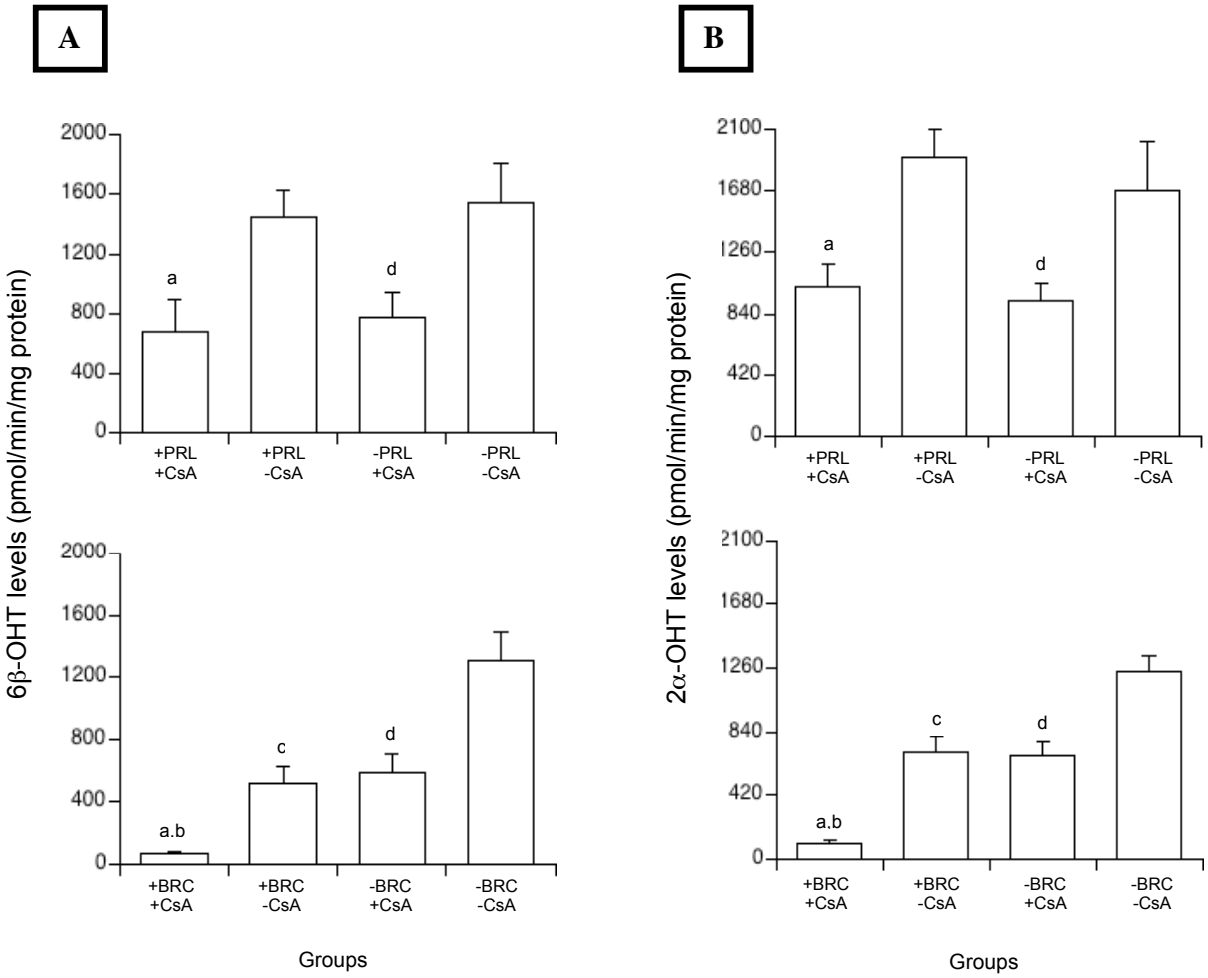


Figure 3.3

Panel A: *In vitro* testosterone hydroxylation by hepatic microsomes following 14 days of treatment. Groups were given subcutaneous doses of either 15 mg/kg cyclosporine or cyclosporine in combination with prolactin, prolactin vehicle,

bromocriptine, or bromocriptine vehicle. Graphs represent CYP3A1/2 activity in the form of 6β-hydroxytestosterone production. Units for metabolite production are pmol metabolite/min/mg microsomal protein. Panel B: *In vitro* testosterone hydroxylation by hepatic microsomes following 14 days of treatment. Groups were given subcutaneous doses of either 15 mg/kg cyclosporine or cyclosporine in combination with prolactin, prolactin vehicle, bromocriptine, or bromocriptine vehicle. Graphs represent CYP2C11 activity in the form of 2α-hydroxytestosterone production. Units for metabolite production are pmol metabolite/min/mg microsomal protein. For both panels: **a**=P<0.05 between +PRL/+CsA and +PRL/−CsA (top graph) or between +BRC/+CsA and +BRC/−CsA groups (bottom graph), **b**=P<0.05 between +PRL/+CsA and −PRL/+CsA (top graph) or between +BRC/+CsA and −BRC/+CsA groups (bottom graph), **c**=P<0.05 between +BRC/−CsA and −BRC/−CsA groups, **c**=P<0.05 between +BRC/−CsA and −BRC/−CsA groups, **d**=P<0.05 between −PRL/+CsA and −PRL/−CsA (top graph) or between −BRC/+CsA and −BRC/−CsA groups (bottom graph).

Figure 3.4 AUC of prolactin secretory profiles and representative graphs of secretion patterns

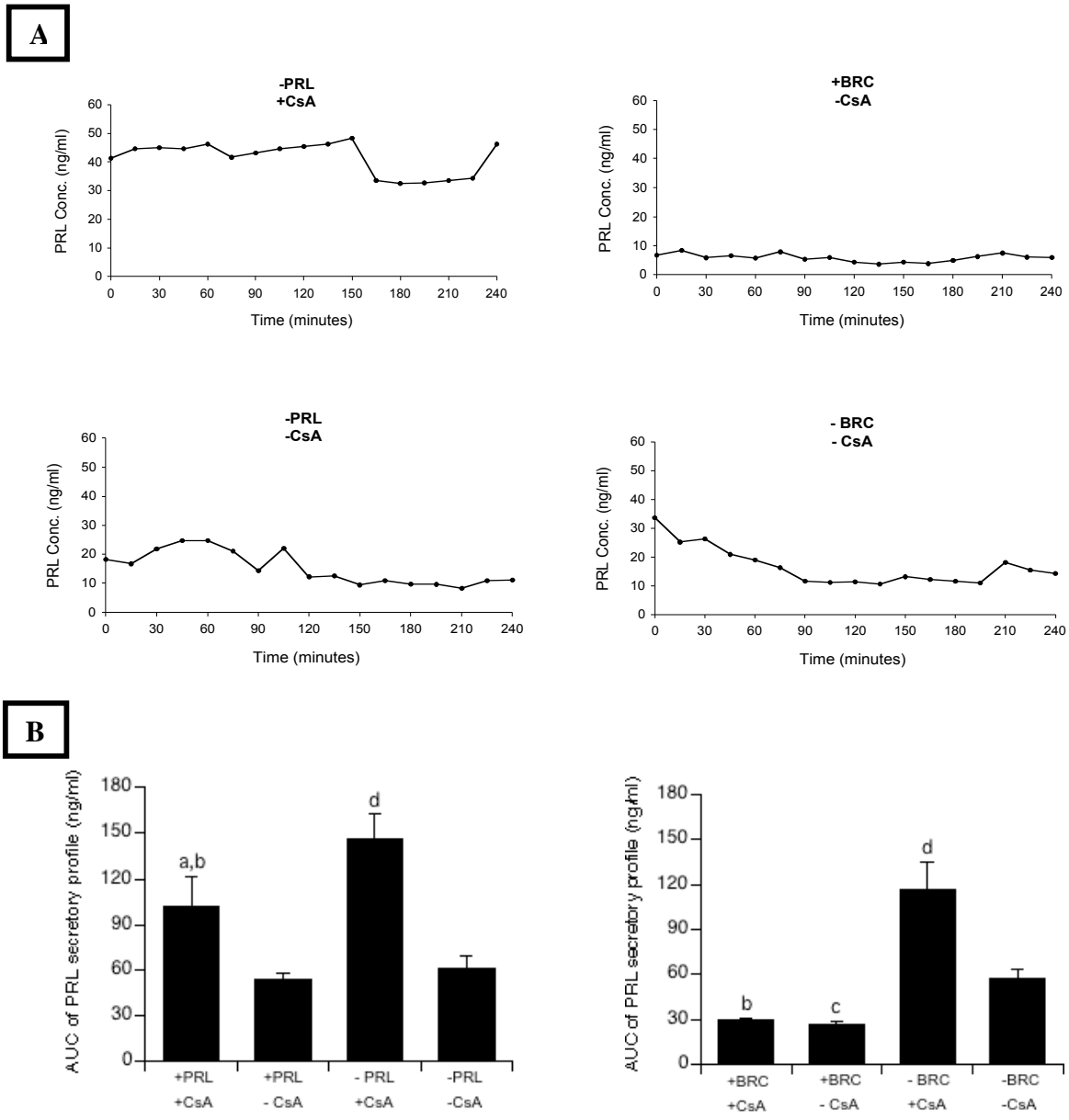


Figure 3.4

Panel A: Representative graphs depicting prolactin secretion profiles of one representative animal from each of the following groups: –PRL/+CsA,

−PRL/−CsA, +BRC/−CsA, −BRC/−CsA, demonstrating the inductive effect of cyclosporine on prolactin secretion and the suppressive effect of bromocriptine, respectively. Blood samples were collected every 15 minutes over a 4-h period. Panel B: Area under the curve calculations of prolactin secretory profiles for circulating serum prolactin in blood samples collected every 15 minutes over a 4-h period. Units are expressed as ng of circulating prolactin per ml of serum. **a**=P<0.05 between +PRL/+CsA and +PRL/−CsA, **b**=P<0.05 between +PRL/+CsA and −PRL/+CsA (left graph) or between +BRC/+CsA and −BRC /+CsA groups (right graph), **c**=P<0.05 between +BRC/−CsA and −BRC/−CsA groups, **d**=P<0.05 between −PRL/+CsA and −PRL/−CsA (left graph) or between −BRC/+CsA and −BRC/−CsA groups (right graph).

Figure 3.5 Effects of re-introduction of prolactin during chronic bromocriptine administration on protein expression andactivity.

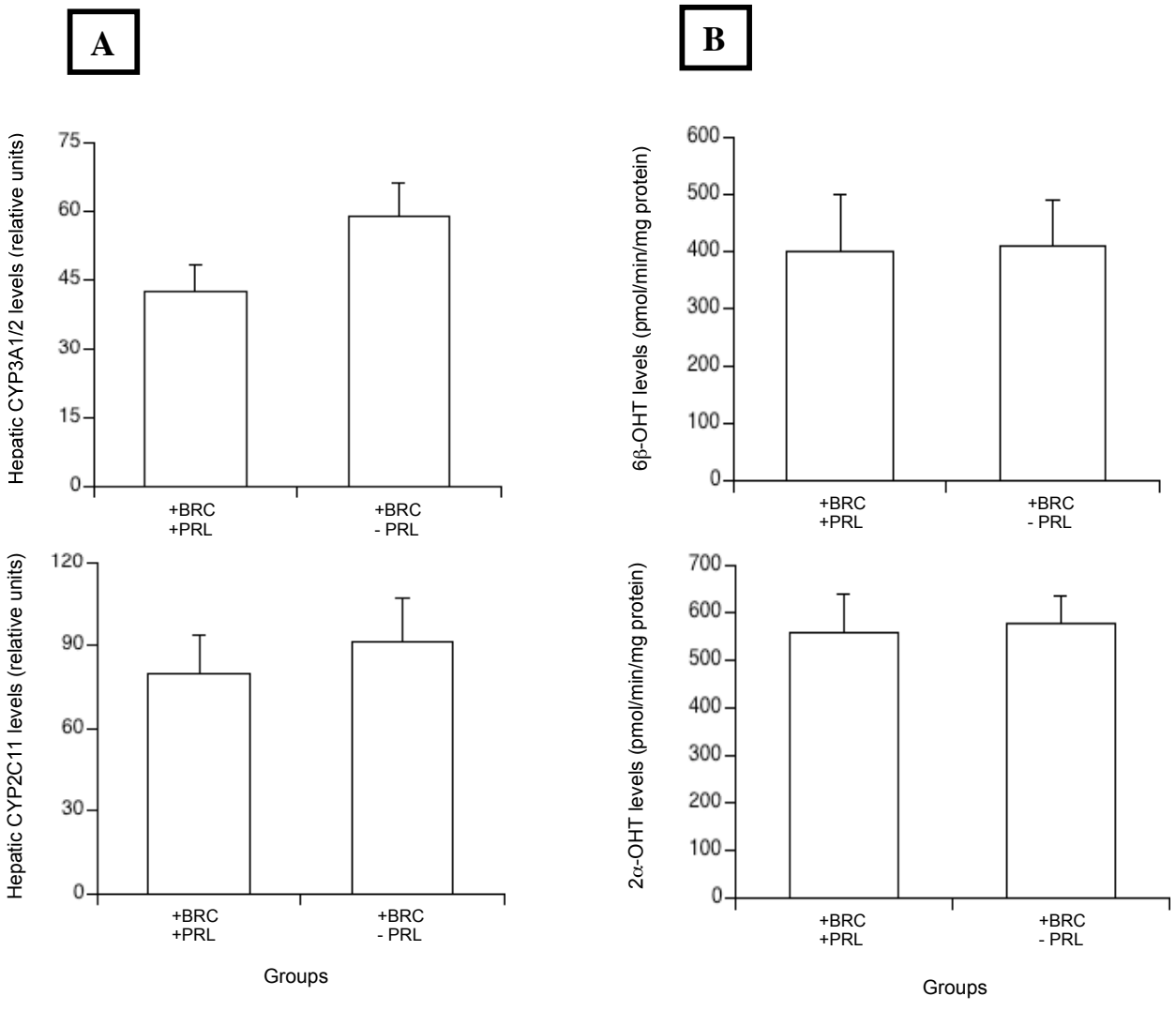


Figure 3.5

Panel A: Western blot analysis of hepatic CYP3A1/2 and CYP2C11 microsomal protein expression in 14-day groups. The two groups were

administered either bromocriptine with prolactin or bromocriptine with prolactin vehicle. All values are expressed as a percent density of the protein standard used for each blot. *=P<0.01 between +BRC/+PRL and +BRC/-P groups. Panel B: *In vitro* testosterone hydroxylation by hepatic microsomes following 14 days of treatment. The two groups were administered either bromocriptine with prolactin or bromocriptine with prolactin vehicle. Graphs represent CYP3A1/2 activity in the form of 6β-hydroxytestosterone production and CYP2C11 activity in the form of 2α-hydroxytestosterone production. Units for metabolite production are pmol metabolite/min/mg microsomal protein.

Figure 3.6 Bromocriptine co-incubation with testosterone competed for CYP3A- but not CYP2C11-catalyzed activity.

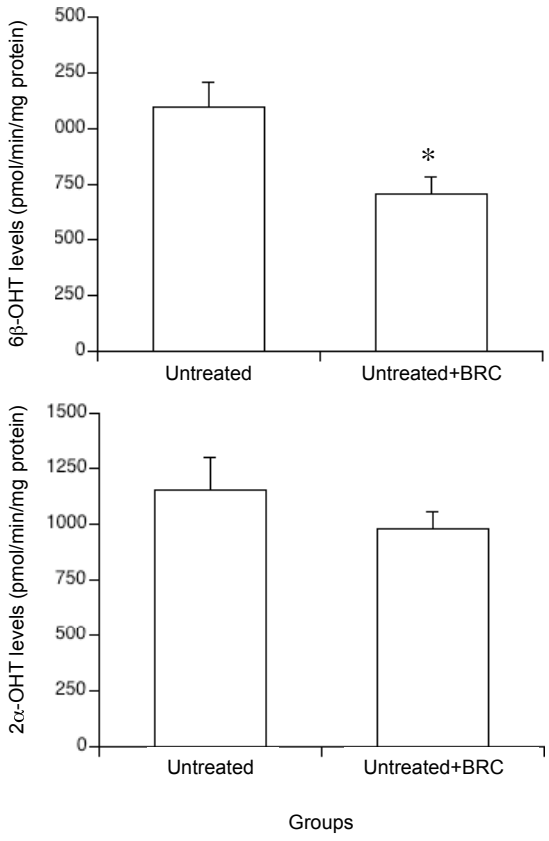


Figure 3.6

Bromocriptine was co-incubated with testosterone and hepatic microsomes in the *in vitro* testosterone hydroxylation assay. Graphs represent CYP3A1/2 activity in the form of 6β-hydroxytestosterone production and CYP2C11 activity in the form of 2α-hydroxytestosterone production. Units for metabolite production are pmol metabolite/min/mg microsomal protein. *=P<0.05.

CHAPTER FOUR

Up-regulation of the JAK/Stat Pathway by Cyclosporine in Primary Rat Hepatocytes

4.1 INTRODUCTION

Cyclosporine (CsA) is a lipophilic immunosuppressant that is commonly administered to patients to prevent immunologic rejection of donor organs following organ transplantation. CsA produced promising results in the clinical setting, however, its usefulness was offset by its side effects. Although one of the most limiting side effects is the onset of organ toxicity, the ability of CsA to suppress cytochrome P450 (P450) enzymes also poses significant problems since CsA is mainly metabolized by a P450 enzyme subfamily, CYP3A (Prueksaritanont et al., 1993b). Because of this dual relationship between CsA and P450, as the expression of P450 decreases, the metabolism of CsA decreases and thus the levels of CsA in the body increases along with the potential for toxic events.

Chronic treatment with moderate daily doses of CsA suppresses protein levels and catalytic activity of the CYP3A2 and CYP2C11 isoforms in the rat (Brunner et al., 1995). Furthermore, this inhibition occurs in a time-dependent (Brunner et al., 1998a) and dose-dependent (Brunner et al., 2000) manner. We previously hypothesized that this suppression was mediated by growth hormone (GH) or prolactin (PRL). Our studies have shown that neither altering GH levels (Lu et al., 2003a) nor modifying PRL levels (Lu et al., in review) affected the extent of suppression of CYP3A and CYP2C11 by CsA, thus suggesting that these two hormones are not direct mediating factors. However, because drug treatment in cell cultures have been shown to directly affect downstream factors and induce changes in protein expression (Chen et al., 1999; von Laue et al., 2000), we decided to investigate the Janus kinase (JAK)/signal transducers and activators of transcription (Stat) pathway as it is the signaling pathway that is immediately responsible for activation of gene expression leading to select P450 protein production.

GH is one of numerous cytokines that can bind to cell surface receptors and initiate a cascade of events leading to the regulation or modification of biological actions. The binding of GH and subsequent signaling within the cell is responsible for several crucial physiological outcomes including bone growth and maintenance of homeostasis (Sims et al., 2000), as well as maintenance of drug metabolism. From as early as 1973, the regulation of hepatic drug

metabolizing enzymes in the rat was shown to be dependent on GH (Wilson, 1973). The sex-specific secretion patterns of GH are critical in the maintenance of the male-specific P450, CYP2C11 (Legraverend et al., 1992; Waxman et al., 1991). When the female pattern of continuous GH secretion was administered to male rats, the male rats then displayed female-specific P450 expression and vice versa (Mode et al., 1989; Pampori and Shapiro, 1996).

Because the GH receptor lacks intrinsic tyrosine kinase activity, the action of GH requires the activation of the JAK/Stat pathway to mediate its intracellular responses. JAK2 is one member of the Janus kinase family of tyrosine kinases that includes JAK1, JAK2, JAK3, and TYK2. It is approximately 130 kD in size and is recruited to the site of GH binding once one GH molecule has bound two growth hormone receptors (GHR) (Cunningham et al., 1991). Two JAK proteins will each bind one of the GHR and proceed to phosphorylate the GHRs and transphosphorylate each other in order to attract and activate signal transducers and activators of transcription (Stats) (Carter-Su and Smit, 1998). This allows for Stats to either homo- or heterodimerize and translocate to the nucleus where they bind to specific sites in the promoter regions of cytokine responsive genes (Shuai et al., 1993).

Stat5b has been demonstrated to be rapidly responsive to the pulsatile pattern of GH secretion in the male rat liver (Waxman et al., 1995b). Conversely, the more continuous secretion of GH seen in adult female rats

results in a low steady-state level of active Stat5b (Gebert et al., 1999). In addition to endogenous factors, Stats have been reported to be responsive to exogenous chemical agents such as ethanol (Chen et al., 1999), dexamethasone (von Laue et al., 2000), and curcumin (Bharti et al., 2003).

For this study, we investigated the effect of acute CsA administration and subsequent treatment with GH on the expressions of CYP3A1/2, CYP2C11 and how these relate to the levels of JAK2 and active Stat protein present in primary rat hepatocytes. Our results indicate that CsA induces JAK2 levels at specific concentrations and Stat5b binding activity increases with increasing concentrations of CsA. This induction correlated with CYP2C11 expression, but not CYP3A1/2.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Unless otherwise noted, all chemicals were purchased in the highest purity available from EM Science (Gibbstown, NJ, USA). CsA was generously provided by Novartis (East Hanover, NJ, USA) in powder form and dissolved in pure ethanol (Aaper Alcohol, Shelbyville, KY, USA) for the study. Purified rat GH was generously provided by the National Hormone & Pituitary Program

under the National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK, Torrance, CA, USA). The anesthetic mixture used in the isolation of rat hepatocytes consisted of a 1:1:1 (v:v:v) ratio of ketamine (100 mg/ml), xylazine (20 mg/ml), and acepromazine (10 mg/ml). Ketamine and xylazine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acepromazine was purchased from Fort Dodge Laboratories, Inc. (Fort Dodge, LA, USA). Materials used in the preparation of the cellular extracts including aprotinin, leupeptin, pepstatin A, DTT, phenylmethylsulfonyl fluoride (PMSF), β-glycerophosphate, sodium orthovanadate, EDTA, EGTA, NP-40, and sodium fluoride were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Pefabloc® SC was purchased from Roche Applied Science (Indianapolis, IN, USA). All media solutions and reagents used in the isolation of primary hepatocytes were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). The Matrigel™ matrix cell culture plates and growth factor reduced Matrigel™ matrix were manufactured by BD Biosciences (Bedford, MA, USA). Reagents used in protein concentration determination were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

JAK2 and Stat5b antibodies were obtained from Upstate (Charlottesville, VA, USA). CYP3A and CYP2C11 antibodies were purchased from Gentest Corporation (Woburn, MA, USA). Rat CYP3A1/2 antibody was isolated from goats that were immunized with CYP3A2 purified from rat liver.

Due to the polyclonal nature of the antiserum, two indistinguishable bands (CYP3A1 and CYP3A2) are detected and are therefore collectively referred to as CYP3A1/2. The microsomal standard used for the relative quantitation of CYP3A1/2 protein was composed of phenobarbital-treated rat liver microsomes (Xenotech, Lenexa, KS, USA). Rat CYP2C11 antibody is also polyclonal and raised in goats, however the cross reactivity with CYP2C13 can be readily distinguished from CYP2C11 based on mobility. CYP2C11 standard was composed of dexamethasone-treated male rat liver microsomes (Xenotech, Lenexa, KS, USA). Goat anti-rabbit-HRP secondary antibody was purchased from Cell Signaling Technology (Beverly, MA). The chemiluminescent substrate used for Western blotting was Supersignal West Pico from Pierce Biotechnology (Rockford, IL, USA).

4.2.2 Isolation of Primary Hepatocytes

Primary rat hepatocytes were isolated based on a modified two-step collagenase method from Seglen (Seglen, 1976). Briefly, male Sprague-Dawley rats between the weights of 285 g and 315 g were used for the study. Once the animals were anesthetized and the hepatic portal vein catheterized, a peristaltic pump (Harvard Apparatus, Inc., Holliston, MA, USA) was used to infuse perfusion buffer supplemented with 2% penicillin/streptomycin. When 350 ml of perfusion buffer was completely perfused, perfusion of an equal volume of digest buffer supplemented with 5% fetal bovine serum (FBS) was initiated.

Both buffers were maintained at a constant temperature of 39° C in an external water bath.

The excised liver was transported to a sterile culture hood and digested further with a scalpel and forceps and then pressed through a mesh screen. Following centrifugation, the cells were spun through a Percoll gradient to remove excess dead material. The cells were counted using the Trypan blue exclusion method and generally displayed a cell viability of between 60-80%.

4.2.3 Cell culture

Once the cells were counted, they were plated on 100 mm thin-layer Matrigel plates at a density of 1.5 X 10^7 cells/plate in Williams' E medium (WEM) supplemented with 1% penicillin/streptomycin, 1% L-glutamine, 5% FBS, 5 ml ITS+ premix (BD Biosciences, Bedford, MA, USA), 5 mM HEPES, and 110 μM dexamethasone. The cultures were maintained at 37° C and 5% CO_2. Following the first 3.5 hours of plating on the day of isolation, the previous media was removed and maintenance medium consisting of WEM supplemented with 1% penicillin/streptomycin and 1% L-glutamine and Matrigel overlay was added to the cultures at a total of 2 mg per 10 ml of medium. The media was replaced each day, however, the Matrigel overlay was not replaced since previous reports have shown that replacing the Matrigel on a daily basis was not advantageous in obtaining phenobarbital-inducible P450 expression (Sidhu et al., 1993). Due to the nature of our P450 study, no other

chemical agents were used in the medium in order to avoid artificial induction of the drug metabolizing enzymes prior to drug treatment.

4.2.4 Drug preparation and treatment

A stock solution of CsA at a concentration of 100 mg/ml was prepared by dissolving CsA powder in pure ethanol. The stock solution was diluted to the following concentrations: 1, 5, and 10 mg/ml. The treatment groups consisted of the following concentrations on plate: 0, 1, 5, 10 μg/ml and contained less than 0.1% of ethanol in each medium preparation. CsA was added to the medium 24 hours following initial plating. The plates were incubated for an additional 24 hours. In groups that included GH treatment, purified rat GH was reconstituted in sterile PBS with 0.1% BSA. GH was placed in the media at a concentration of 300 ng/ml (based on reports of *in vivo* GH levels reaching between 250 and 300 ng/ml) and remained on the plates for 2 hours until cells were removed from the plates (Waxman et al., 1991).

4.2.5 Preparation of cellular extracts

Cells were washed once with sterile PBS and then collected using two rinses with ice-cold sterile PBS. The entire cell and Matrigel matrix were scraped off the plates with a cell scraper and placed into conical tubes for centrifugation. Cells were centrifuged at 2000 X g at 4^{o} C for 5 minutes. Immediately following the spin, the supernatant was removed and 200 μl of ice-

cold lysis buffer containing protease inhibitors was added to the tube. The pellet and lysis buffer were repeatedly drawn and expelled through a 20-gauge needle to lyse the cells. The cells were then incubated on ice for 40 mins. After incubating on ice, the samples were centrifuged at 14,000 X g for 20 mins. at 4° C. The supernatant containing the cellular extracts was collected and stored at –80° C until analysis.

4.2.6 Cell proliferation assay

The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) was used to determine the relative number of viable cells in cell preparations containing CsA in order to assess the relative level of cytotoxicity of various concentrations of the drug. The assay is composed of two solutions: a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS). When MTS is encountered by living cells, it is bioreduced into a colored formazan product with the aid of dehydrogenase enzymes found in active cells. The MTS assay has been tested against others including XTT (Goodwin et al., 1995) and MTT (Riss and Moravec, 1992) and the MTS system proved to be the least labor-intensive and most stable for use.

Cells were counted and plated on a 96-well plate at a density of 1.5 X 10^5 cells/well in WEM supplemented with 5% penicillin/streptomycin, 5% L-

glutamine, 5% FBS, 5 ml ITS+ premix (BD Biosciences, Bedford, MA, USA), 5 mM HEPES, and 110 μM dexamethasone. They were incubated at 37^{o} C and 5% CO_2 for 3.5 hours. Then, the media was changed to WEM supplemented with only 5% penicillin/streptomycin and 5% L-glutamine. Following 24 hours of incubation, CsA was added to the cultures at the following final concentrations on cells (in quadruplicate): 0, 1, 5, 10 μg/ml. At the end of the incubation period, 20 μl of the MTS/PMS mixture was added to each well. The cells were incubated for an additional 2 hours, following which the plate was read on a 96-well plate reader at a wavelength of 490 nm.

4.2.7 Gel electrophoresis and immunoblot analysis

SDS-PAGE was performed as previously described (Laemmli, 1970) using an 8% polyacrylamide separating gel. Protein on the gel was transferred to nitrocellulose sheets by a previously described method (Schnier et al., 1989b). After protein transfer, the nitrocellulose sheets were blocked with 5% non-fat dry milk (NFDM) in Tris-buffered saline (TBS) at room temperature. JAK2 and Stat5b were incubated in 5% NFDM for 1 hour and overnight in primary antibody at a concentration of 1:1500. The blots were placed in secondary antibody for approximately 2 hours and at a concentration of 1:20,000. The P450 blots were incubated in NFDM overnight and in primary antibody at a concentration of 1:3000 for a maximum of 1.5 hours. Incubation with secondary antibody (1:3000) lasted for approximately 2 hours. Immune

complexes for CYP3A1/2 and CYP2C11 were detected with an NEN chemilumnescence reagent kit as described by the manufacturer (New England Nuclear Life Science Products, Boston, MA, USA). JAK2 and Stat5b were detected using Supersignal West Pico chemiluminescent substrate. Blot densities were measured using a flatbed scanner (Microtek, Hsinchu, Taiwan) and analyzed on a Dell PC computer using the Kodak 1D image analysis software, version 3.5 (Eastman Kodak Co., Rochester, NY, USA).

4.2.8 TransAM Stat family transcription factor assay kit

A transcription factor assay kit from Active Motif (Carlsbad, CA, USA) was used to detect activated Stat5b proteins in cellular extracts. The kit uses a 96-well plate coated with a consensus sequence oligonucleotide that corresponds to Stats. After the sample is loaded into the well, the sample containing active Stats is allowed to bind to the oligonucleotides and form a complex. An antibody directed specifically against Stat5b is added to the wells which then binds to only Stat5b present in the cells. After a wash step, only Stat5b protein that is bound to both the oligonucleotide and to the Stat5b antibody remains on the plate. Thus, only the presence of active Stat5b with binding ability is measured. A non-specific secondary antibody containing a horseradish-peroxidase component is added to the wells along with a colorimetric agent. The plate is then read spectrophotometrically at a wavelength of 450 nm with a reference wavelength of 655 nm.

4.2.9 Data Analysis

Sample densities from Western blots were compared with standard microsomal protein densities and expressed as a percentage. One-way ANOVA and a priori means comparisons tests were employed using respective vehicle groups as the control with the aid of the SuperANOVA statistical program (Abacus Concepts, Inc., Berkeley, CA, USA). Data are presented as mean ± standard error. When the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$), the differences were then considered to be statistically significant.

4.3 RESULTS

4.3.1 Short-term exposure to CsA is not cytotoxic to primary rat hepatocytes

Blood levels of rats receiving chronic CsA therapy at 15 mg/kg/day was determined by our laboratory to be between 3000 to 5500 ng/ml (unpublished data). These levels were sufficient enough to cause significant suppressions of CYP3A1/2 and CYP2C11 protein and activity. The selection of the concentrations of CsA at 0, 1, 5, and 10 μg/ml were based on our previous data and the intent to investigate the effect of therapeutic as well as supratherapeutic concentrations of CsA. An MTS assay was conducted to assess the relative

cytotoxicity of CsA on primary rat hepatocytes. Figure 4.1 shows that the level of cell proliferation in the cells that underwent no treatment whatsoever did not significantly differ from the ethanol-treated (0 μg/ml) cells, indicating that the concentration of ethanol added to cultures in this study (0.1%) is not cytotoxic to hepatocytes. In addition, there were no decreases in cell proliferation below the IC_{50} of the non-treated cells, thus indicating that after 24 hours of treatment, 1, 5, and 10 μg/ml of CsA did not have a toxic effect on rat hepatocytes.

4.3.2 JAK2 expression is induced by CsA in a dose-dependent manner

When CsA was added to cultures of primary rat hepatocytes at a concentration of 1 μg/ml, there was a modest decrease in JAK2 expression as demonstrated through Western blots (Figure 4.2). However, at 5 μg/ml, there was a significant increase in JAK2 expression ($p<0.001$). The addition of GH to these cultures did not cause a statistically significant change in levels of JAK2 at any concentration of CsA. However, a significant decrease in JAK2 expression was exhibited in cells that were administered both CsA and GH as compared with CsA alone. At the 0 and 5 μg/ml levels of CsA, the addition of GH substantially lowered JAK2 protein when compared with non-GH-treated cells ($p<0.05$; $p<0.01$, respectively).

4.3.3 Suppression of CYP3A1/2 by CsA is dose-dependent in hepatocytes

Previous findings in our lab have shown that CYP3A1/2 is significantly suppressed by CsA in vivo beginning at a concentration of 5 mg/kg/day when given orally (Brunner et al., 2000) and 15 mg/kg/day subcutaneously (Lu et al., 2003a). In this in vitro study, CYP3A1/2 protein was significantly reduced only at the 5 μg/ml concentration of CsA as compared with the zero control ($p<0.05$, Figure 4.3). Although both 1 μg/ml and 10 μg/ml of CsA caused a decrease in protein expression, the differences were not statistically significant. To date, few studies in literature have examined suppressive effects of CYP3A1/2 in primary rat hepatocytes as a result of drug treatment. This may be due in part to the difficulties in maintaining P450 expression in culture. However, when freshly isolated rat hepatocytes are maintained on thin-layer Matrigel plates with a Matrigel overlay, the mRNA and protein expressions of some forms of P450 including CYP3A1/2 are constitutively expressed along with other isoforms of P450s that are inducible (Kocarek et al., 1992; Lecluyse et al., 1999; Sidhu et al., 1993). Using this Matrigel overlay method and WEM for our culture medium, we were able to obtain a basal level of CYP3A expression in short-term culture.

4.3.4 Changes in CYP2C11 protein expression correlate with Stat5b DNA-binding activity

According to Western blotting results obtained from this study, in vitro treatment of CsA at 10 μg/ml to primary hepatocytes cultures causes a slight increase in CYP2C11 protein expression ($p<0.05$, Figure 4.4). This coincided with a pattern of increasing Stat5b binding activity that includes a 25% increase at both the 5 and 10 μg/ml concentrations of CsA ($p<0.05$; $p<0.05$, Figure 4.5). In the presence of GH, Stat5b binding activity was significantly decreased at the same concentrations that increased activity when CsA alone was added ($p<0.05$; $p<0.01$, respectively). CYP2C11 followed the same trend, however the decreases were not statistically significant. When GH-treated and non-GH-treated cultures were compared with each other, significant suppressive effects on both Stat5b activity and CYP2C11 protein expression were caused by GH at all concentrations of CsA beginning at 1 μg/ml (1 μg/ml, $p<0.01$; 5 μg/ml, $p<0.01$; 10 μg/ml, $p<0.01$).

4.3.5 CsA caused an increase in Stat5b oligonucleotide-binding activity without changing Stat5b expression

Using an oligonucleotide-binding assay, we were able to determine the relative DNA-binding capacity, or activity, of each sample of cellular extracts. The results show that Stat5b activity increased with increasing concentrations

of CsA, with the most significant inductions at 5 and 10 μg/ml ($p<0.05$; $p<0.05$, Figure 4.5). When GH was added to these cultures, the activity of Stat5b declined as the concentration of CsA increased. Again, the greatest differences were detected at the 5 μg/ml and 10 μg/ml concentrations ($p<0.01$; $p<0.05$, respectively). The addition of GH significantly decreased the activity of Stat5b when compared with the respective no-hormone controls only in cells that were treated with CsA (1 μg/ml, $p<0.01$; 5 μg/ml, $p<0.01$; 10 μg/ml, $p<0.01$). CsA did not significantly alter Stat5b protein expression as determined by Western blotting at any concentration either in the presence of GH or not. Although GH-treated cells exhibited a higher expression of Stat5b than their respective non-GH-treated controls, the differences were not statistically significant. In accordance to previous reports demonstrated in CWSV-1 cells, Stat5b travels down a gel as a set of multiple bands in Western blots using a Stat5b-specific antibody (Gebert et al., 1997). A third band above the lowest two bands was determined to be serine-phosphorylated Stat5b with a fourth band appearing in response to GH treatment. EMSA tests conducted in the same previous study confirmed that the uppermost band that appeared following GH treatment is tyrosine phosphorylated. In the present study, the authors found the same bands that appeared in samples both with and without GH treatment (Figure 4.6).

4.4 DISCUSSION

Although the depressive effects of CsA on hepatic drug metabolism have been studied extensively *in vivo*, few studies have examined the molecular mechanisms of these suppressions *in vitro*. Studies investigating the mechanistic cause are crucial because of CsA's widely known adverse effects on organ toxicity and the potential for these effects to be a function of CsA's metabolism. Since CYP3A2 is the primary P450 isoform responsible for CsA metabolism in the liver of male rats and CsA also suppresses this enzyme, this isoform was of chief importance. In addition to suppressing the male-dominant CYP3A2, CYP2C11, a male-specific isoform, is also suppressed by CsA *in vivo*. Because normal levels of CYP2C11 and CYP3A2 protein are obligatorily dependent on the pulsatile GH secretion profile found in male rats (Waxman et al., 1991; Waxman et al., 1995a), our preliminary hypothesis was to study GH as a possible mediator in the CsA-induced suppression of these isoforms. We have shown that altering GH levels did not lead to a significant change in the level of suppression caused by CsA (Lu et al., 2003a). Thus, CsA is likely altering levels of P450 via a different mechanism. Since the JAK/Stat pathway has been suggested to be responsible for the sexual dimorphism in male liver gene expression (Waxman et al., 1995b), the aim of the present study was to assess the effects of CsA on this pathway and potential consequences on P450

expression as well as to investigate the *in vitro* effect of the presence of GH in conjunction with CsA.

In the present study, a relatively naïve medium was used to maintain the freshly isolated hepatocytes. The biphasic inductive and suppressive effects of glucocorticoids on P450 expression have been well-established in hepatocyte cultures (Iber et al., 1997; Sidhu and Omiecinski, 1995), where low concentrations of dexamethasone (DEX) ($<10^{-8}$ M) induce CYP2C11 and high concentrations ($>10^{-7}$ M) suppress it. Because of this potential for artificial induction of P450 expression, we did not include the use of DEX in our maintenance medium in order to avoid a masking effect on even slight changes in enzyme expression. Thus, every other aspect of the isolation process and culturing conditions were optimized for maximal P450 expression. Matrigel was chosen for the substratum as it maintains higher overall P450 expressions for longer periods of time (Kocarek et al., 1992) and displays higher CYP3A1/2 basal expression when the concentration of DEX is low (Silva et al., 1998). The use of an extracellular overlay in hepatocyte cultures is also beneficial for the expression of P450s (Lecluyse et al., 1999). The addition of insulin was left out in this study since it was found that the expression of CYP3A1 in hepatocytes not exposed to insulin were more responsive to the inductive effects of phenobarbital (Sidhu and Omiecinski, 1999), thus indicating an overall heightened sensitivity to xenobiotics.

In this study, CsA did not have the same suppressive effects on P450 enzymes that have been previously found in *in vivo* studies. We have previously found that the suppression CYP3A2 is dose-specific in adult male rats, with a suppressive oral dose beginning at 5 mg/kg (Brunner et al., 2000). However, CsA only significantly decreased the expression of CYP3A1/2 *in vitro* at a concentration of 5 μg/ml, indicating a dose-dependent effect that appears to be suppressive at the low to medium concentrations while not having an effect at a higher concentration. Since concentrations higher than 10 μg/ml were not tested in this study, it is not known at this time whether higher concentrations may be inductive on protein expression, as in the case of dexamethasone. Since one of the known avenues of CsA's entry into hepatocytes is by way of passive diffusion (Fricker and Fahr, 1997), it is unlikely that there would be a receptor saturation effect regarding uptake of CsA into liver cells.

The present study demonstrated an *in vitro* correlation between Stat5b binding activity and CYP2C11 protein expression. Studies in hypophysectomized rats have shown that twice daily injections of exogenous GH is associated with a coordinate increase in Stat5b binding activity (Waxman et al., 1995b). It has also been established that Stat5b is the vital determinant of sexual dimorphic liver gene expression induced by GH (Davey et al., 1999). Thus, it is not surprising that the levels of CYP2C11 protein expression in this study corresponded with the level of DNA-binding activity of Stat5b. However,

while CsA is suppressive to CYP2C11 levels *in vivo*, it appears to be inductive *in vitro*. At the highest concentration of CsA, CYP2C11 was induced by 30% over the vehicle (zero) control. Correspondingly, Stat5b activity was induced at the two highest concentrations of CsA. The cause for this *in vitro* induction is not known at this time, although it stresses the importance of recognizing differences in the regulation of drug metabolism in cell culture as opposed to *in vivo* conditions. The addition of exogenous GH had the opposite effect. As the concentration of CsA increased, GH lowered the levels of Stat5b activity and CYP2C11. The depressive effect of the excess GH emphasizes not only that hormonal status overrides the effects of CsA, but also that elevated basal levels of GH are detrimental in the proper expression of CYP2C11. Proper maintenance of CYP2C11 levels requires an "on/off" pattern of GH secretion, where the duration of the "off" period is critical (Waxman et al., 1991). Thus, having excess GH available to cells likely caused a down-regulation of P450s.

The exact mechanism behind the discordance between Stat5b binding activity and Stat5b protein levels found for this study is unknown. Time courses of GH treatment to hyposectomized rats generally increase Stat5b protein levels and activity during the first hour (Gebert et al., 1997). However, in this study, when a constant level of GH was present in conjunction with CsA, Stat5b protein expression increased over the levels with only CsA present in the media, indicating that CsA may be interfering with the down-regulation of

Stat5b protein that is normally seen with supraphysiological levels of GH. Stat5b oligonucleotide binding activity did decrease steadily with increasing concentrations of CsA in the presence of GH.

Although literature evidence investigating CsA's effect on the JAK/Stat pathway is scarce, one study did discover that CsA can eliminate the induction of Stats caused by IL-6 (Henttinen et al., 1995). Because CsA can suppress T-cell mediated immune responses, it was determined that the induction of Stats in the study by IL-6 was mediated by a secondary factor produced by T-cells. In light of this evidence, the significant increase in binding activity caused by CsA treatment in the present study indicates that this event is likely not mediated by T-cells. Furthermore, CsA may have a suppressive influence on negative regulators of the JAK/Stat pathway, such as suppressors of cytokine signaling (SOCS), that leads to an upregulation of CYP2C11 *in vitro*. This differentially regulated *in vitro*/*in vivo* effect on CYP2C11 is the first to be reported for CsA.

CsA had a dose-dependent inductive effect on JAK2 expression where JAK2 was induced at a concentration of 5 μg/ml. Because this trend did not correspond with CYP2C11 levels, there may be other signaling pathways responsible for CsA-induced changes in CYP2C11 expression. Much evidence has suggested a role for cross-talk between Stats and various factors including ERK (Krasilnikov et al., 2003), ERs (Faulds et al., 2001), and PI3-K (Kirito et al., 2002). This interrelationship with the JAK/Stat pathway can lead to external

influence over the outcome of this intracellular signaling mechanism. Moreover, because the cells were not assayed until well after the drug treatment period, it is possible that down-regulation of JAK2 had taken place in the cell while activated Stat5b remained. In addition, CYP3A1/2 does not correlate with JAK2 expression or Stat5b activity, indicating that CsA's effects on this isoform is not dependent on the JAK/Stat pathway.

CsA has undoubtedly contributed to the success of organ transplantation. Because patients receiving organ allografts are on immunosuppressant therapy for the remainder of their lives, ongoing research in this area is crucial. Taken together, this study relates an increase in *in vitro* CYP2C11 levels with and increase in Stat5b binding activity caused by CsA treatment. Thus, this evidence suggests that CsA can directly modify factors involved in GH-initiated intracelluluar signaling to alter the fate of P450 expression *in vitro*. However, given the complexities of immunopharmacology, the endocrine system, and hepatic drug metabolism, further in-depth studies on the distinct pathways and signaling mechanisms involved in this interplay are needed.

Figure 4.1 Effect of CsA on cell proliferation

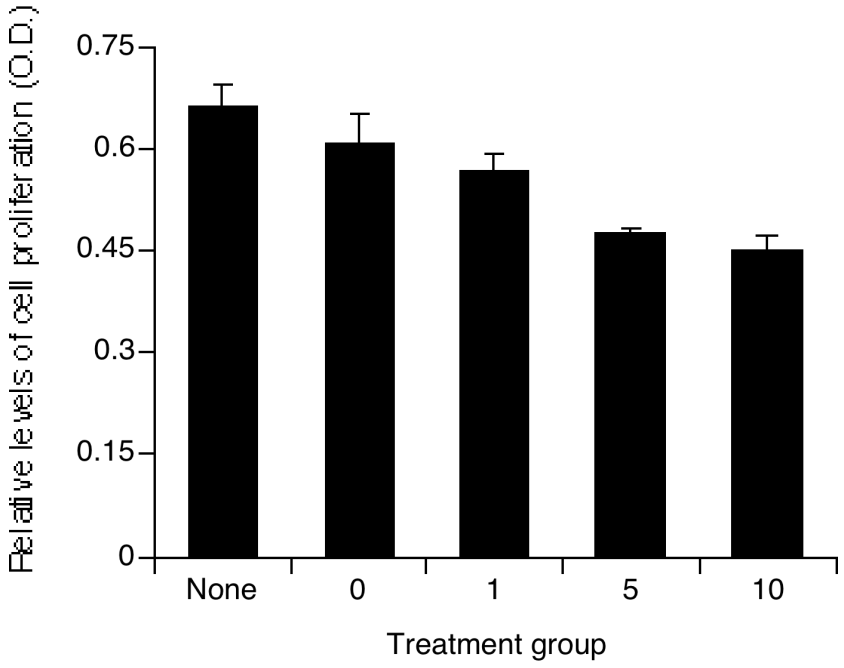


Figure 4.1

The relative cytotoxicity of CsA in primary rat hepatocytes (1.5 x 10^5 cells) was measured using the MTS assay for cell proliferation. Cells were treated with CsA at the following concentrations for 24 hours: 0, 1, 5, and 10 μg/ml. Values are represented as the means of four replicate O.D. values for each treatment level. "None" denotes cells that were not treated with either ethanol vehicle or CsA.

Figure 4.2 JAK2 expression following treatment with CsA and GH

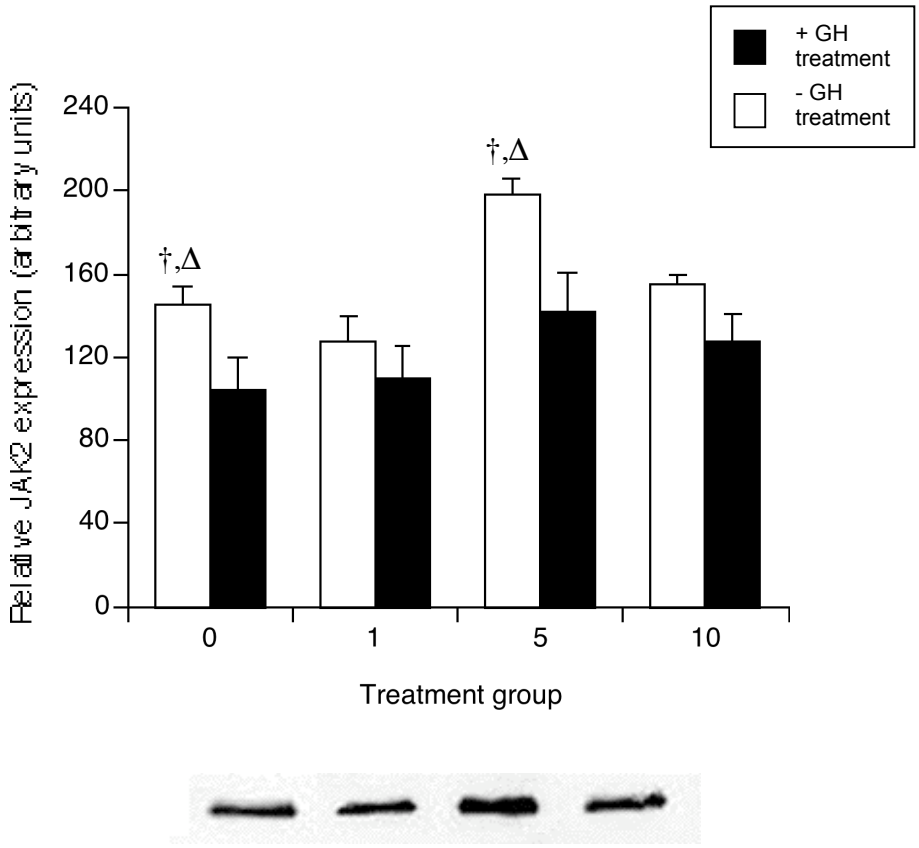


Figure 4.2

The effects of increasing concentrations of CsA and the addition of GH on relative JAK2 expression were determined using Western blotting. Representative blot of JAK2 shows CsA treatment only from left to right: 0, 1, 5, 10 ng/ml. † = $p<0.05$ between white bars bearing this symbol. Δ = $p<0.05$ between the white bar and black bar within the same concentration grouping. Open bar = CsA treatment only. Black bar = CsA + GH treatment.

Figure 4.3 Effect of CsA on CYP3A1/2

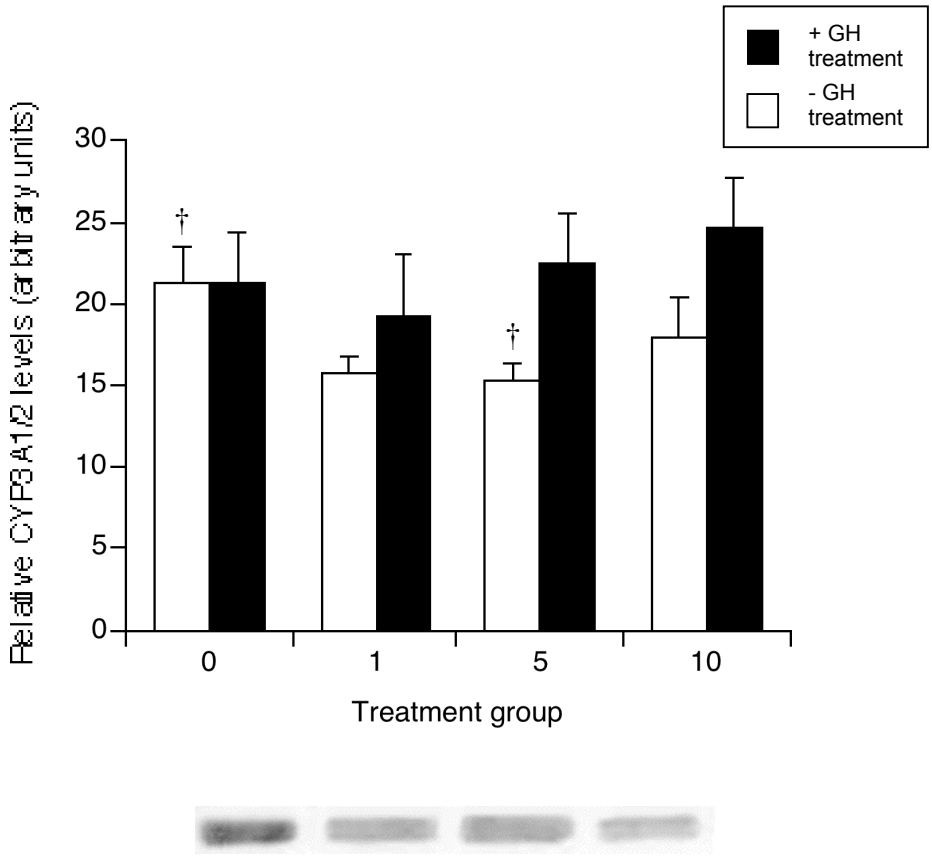


Figure 4.3

The effects of increasing concentrations of CsA and the addition of GH on relative CYP3A1/2 expression were determined using Western blotting. Representative blot of CYP3A1/2 shows CsA treatment only from left to right: 0, 1, 5, 10 ng/ml. † = $p<0.05$ between white bars bearing this symbol. Open bar = CsA treatment only. Black bar = CsA + GH treatment.

Figure 4.4 CYP2C11 expression following CsA and GH treatment

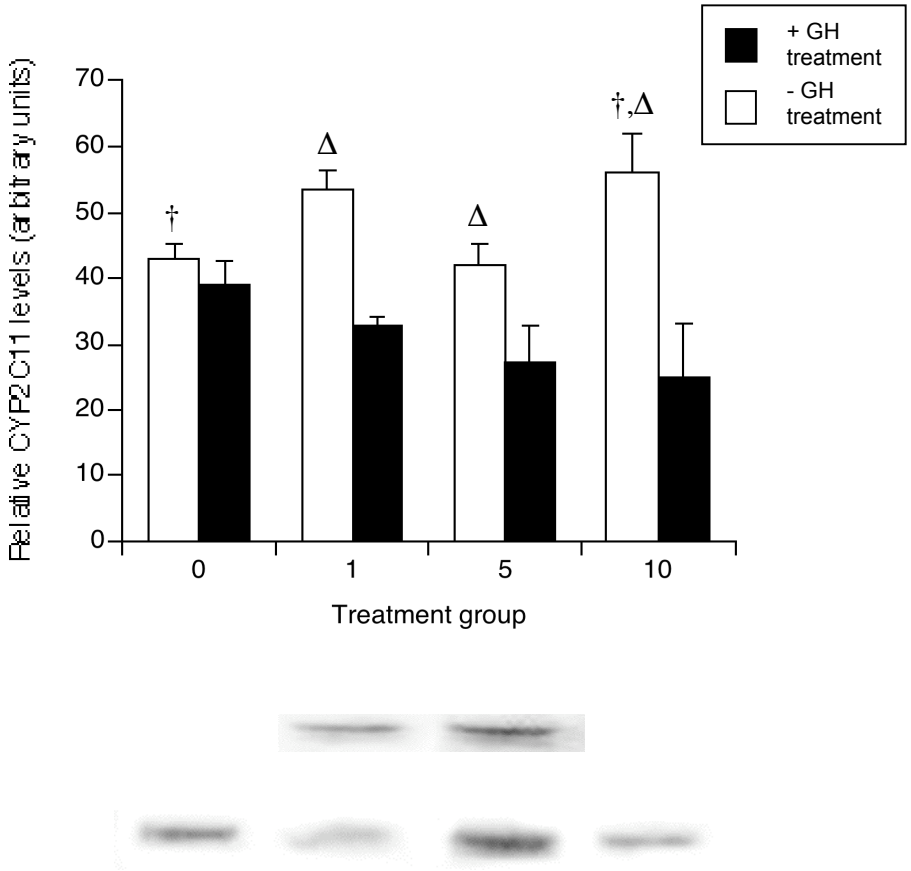


Figure 4.4

The effects of increasing concentrations of CsA and the addition of GH on relative CYP2C11 expression were determined using Western blotting. CYP2C11 blot in top panel from left to right: 0, 10 ng/ml; bottom panel: 5, 5+GH, 10, 10+GH. † = $p<0.05$ between white bars bearing this symbol. Δ = $p<0.05$ between the white bar and black bar within the same concentration grouping. Open bar = CsA treatment only. Black bar = CsA + GH treatment.

Figure 4.5 Stat5b binding activity

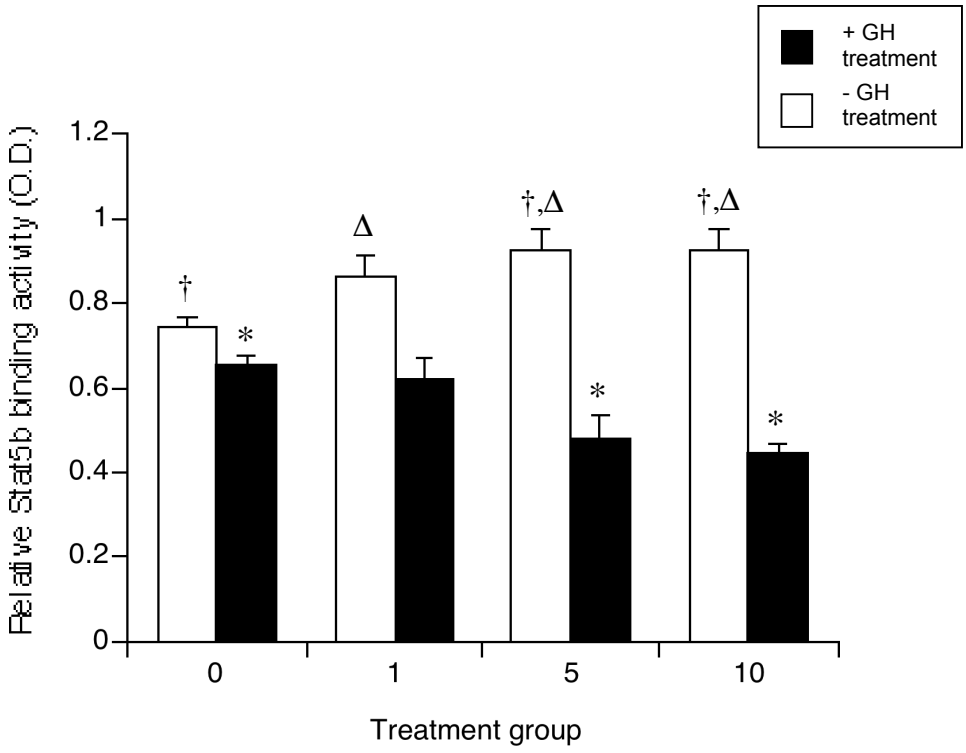


Figure 4.5

Specific Stat5b oligonucleotide binding activity was measured using a TransAM Stat assay kit that uses a Stat consensus sequence and a Stat5b-specific antibody for determining binding capacity. † = $p<0.05$ between white bars bearing this symbol. Δ = $p<0.05$ between the white bar and black bar within the same concentration grouping. * = $p<0.05$ between black bars bearing this symbol. Open bar = CsA treatment only. Black bar = CsA + GH treatment.

Figure 4.6 Stat5b protein expression

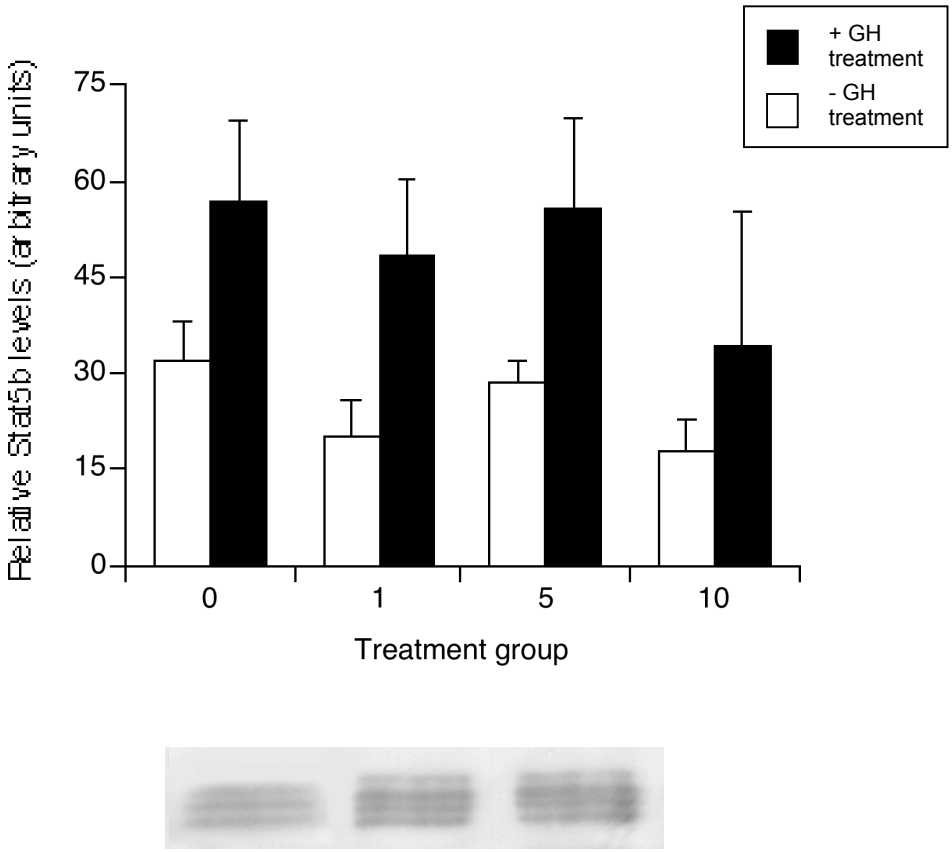


Figure 4.6

Stat5b protein expression was investigated using Western blotting with a specific Stat5b antibody. The proteins traveled as a triplet of bands and in quadruplet when GH was introduced to the cells. From left to right the bands are: 0 ng/ml, 1+GH, 5+GH. There were no statistically significant changes between treatments.

CHAPTER FIVE

Summary and Conclusions

Cyclosporine has had proven success in preventing immunological rejection following organ transplantation. However, as in the case of many potent pioneer drugs, several adverse side effects limit its clinical use. Of all the difficulties associated with its treatment, the lack of a direct correlation between blood levels and efficacy and toxicity remains the most limiting obstacle to overcome. This effect may be caused by several factors including the suppression of P450s in the liver. This event is of chief importance because a subfamily of this monooxygenase system, CYP3A, is the main family of enzymes responsible for the metabolism of cyclosporine. Thus, as cyclosporine is being administered over time, it is limiting its own breakdown. In addition, nephrotoxicity and hepatotoxicity are significant adverse effects that can also occur as a result of chronic cyclosporine therapy. The suppression of P450s may play a role in the underlying causes of these effects as well. The objectives of this work were to investigate hormonal factors as potential mediators in the *in vivo* suppressions of CYP3A1/2 and CYP2C11.

Chronic, moderate daily doses of cyclosporine significantly suppressed the expressions and activities of CYP3A1/2 and CYP2C11. This effect was not attenuated by the administration of excess exogenous growth hormone. When administered in the absence of cyclosporine, growth hormone also suppressed CYP3A1/2 and CYP2C11 protein levels as compared to growth hormone vehicle. In the presence of cyclosporine, growth hormone did not cause a further suppression of either CYP3A1/2 or CYP2C11 expression when compared to cyclosporine treatment with growth hormone vehicle. Testosterone *in vitro* catalytic assays confirmed that cyclosporine and growth hormone separately cause significant decreases in activity levels. Also, the concomitant administration of growth hormone and cyclosporine caused lowered productions of 16α-, 2α-, 6β-, and 2β-OHT as compared to the administration of growth hormone with cyclosporine vehicle and as compared to the administration of growth hormone vehicle with cyclosporine. This study shows that growth hormone is a dominating factor over cyclosporine in determining hepatic P450 expression and activity. Furthermore, cyclosporine does not seem to be altering growth hormone levels as a mediating event in suppressing P450 expression and activity. Since cyclosporine given in combination with growth hormone further suppressed P450 activity as compared with cyclosporine given in combination with vehicle, this suggests that changes in hormonal status is

likely to be only one of the many factors that is responsible for the lack of a clear association between cyclosporine dosing and markers of toxicity.

Prolactin was also investigated as a possible mediating factor in the suppressions of CYP3A1/2 and CYP2C11 by cyclosporine. The administration of prolactin did not significantly alter CYP3A1/2 protein expression by itself nor did it attenuate the suppression caused by cyclosporine. Hypoprolactinemia, produced by bromocriptine, caused a significant suppression of CYP3A1/2 activity levels when bromocriptine was administered alone and in combination with cyclosporine. However, the cause of the suppression by bromocriptine was likely not the result of lowering prolactin levels as determined by an *in vitro* substrate competition assay that revealed that bromocriptine was likely a competitor for or inhibitor of testosterone metabolism by P450s. Thus, bromocriptine not only served to lower prolactin secretion, but it was demonstrated that the drug itself also had a direct inhibitory effect on CYP3A1/2 activity. Bromocriptine administration also lowered CYP2C11 protein expression and activity, while prolactin administration had virtually no effect on CYP2C11. Chronic cyclosporine administration caused a significant increase in prolactin area under the curve. However, while bromocriptine caused an expected decrease in endogenous prolactin secretion, concurrent cyclosporine administration did not recover these levels. Overall, while both cyclosporine and bromocriptine, separately, can significantly alter the fate of

hepatic P450 enzymes, the suppression is likely not due to an alteration in prolactin levels.

A separate *in vitro* study was conducted to examine the mechanism of bromocriptine's effects on CYP3A1/2 activity. The study revealed that bromocriptine is a moderately potent competitive inhibitor of CYP3A-catalyzed testosterone metabolism. Thus, not only should future studies using bromocriptine as an agent to abolish prolactin secretion take into account the consequences on hepatic drug metabolism, but also drug interactions involving CYP3A metabolism should be considered as well.

Because growth hormone and prolactin were found not to be directly involved in the suppression of P450s by cyclosporine, other downstream intracellular signaling factors were investigated as mediators. Results from our *in vitro* studies examining the effects of cyclosporine on the JAK/Stat pathway and subsequent expressions of CYP3A1/2 and CYP2C11 indicate that cyclosporine has differential effects on P450 expressions *in vivo* vs. *in vitro*. While cyclosporine suppresses CYP2C11 *in vivo*, cyclosporine induces CYP2C11 expression in primary rat hepatocytes in a dose-dependent fashion. Although the cause of this is unknown, the binding activity of Stat5b, a transcription factor responsive to growth hormone pulses, correlates closely with the levels of CYP2C11. However, CYP3A1/2 does not, thus indicating that the expression of CYP3A1/2 is not directly modulated by Stat5b.

Altogether, these studies show that cyclosporine-induced suppression of P450s is not a direct result of the alteration of hormone levels. However, our studies in rat hepatocytes indicate that cyclosporine has an effect on intracellular signaling factors that are downstream from growth hormone. Thus, these studies provide a mechanistic foundation for explaining cyclosporine-induced changes in hepatic drug metabolism and aid in the understanding of the complex interrelationship between immunopharmacology, drug metabolism, and hormonal factors.

Future studies investigating the potential connection between CsA and PXR may further clarify the relationship between CsA and P450s. Previous studies have shown that PXR plays a regulatory role in the expression of CYP3A and thus may be involved in the modulation of P450s by CsA (Bertilsson et al., 1998; Kliewer et al., 1998). In addition, future studies that may aid in a more in-depth investigation of CsA's effects on the JAK/Stat pathway should include the use of hepatocytes from hypophysectomized rats. These cells can be induced to express Stat5 in response to GH pulses. Once *in vivo* conditions are simulated using exogenous GH pulses, these cells should be challenged with CsA during a timecourse of GH treatment. This experiment would provide a clearer understanding of the exact effect of CsA during each phase of the GH secretion profile.

APPENDIX I

Bromocriptine is a Selective and Competitive Inhibitor for CYP3A-dependent Testosterone Hydroxylation

A1.1 INTRODUCTION

Bromocriptine (BRC), an ergot alkaloid, is a specific dopamine receptor agonist. It can drastically reduce the secretion of prolactin (PRL), whose main inhibitory influence is dopamine. Due to BRC's ability to rapidly suppress PRL secretion, it has been successfully used to treat a variety of conditions including: hyperprolactinemia (Floersheim-Shachar and Keller, 1977), pituitary adenomas (Scamoni et al., 1991), acromegaly (Wass et al., 1977), and early stage Parkinson's disease (Kondo, 2002; Parkes et al., 1976).

It is known that the majority of BRC's metabolism takes place in the liver. Several lines of evidence suggest that the CYP3A subfamily is the main P450 enzyme responsible for the biotransformation of BRC (Althaus et al., 2000; Matsubayashi et al., 1997; Peyronneau et al., 1994a). More specifically, through the

use of chemical inhibition studies, it was reported that CYP3A4 in humans was the isoform responsible for catalyzing the breakdown of ergot alkaloids similar to BRC (Ball et al., 1992). While BRC is known to be metabolized by CYP3A, studies on the effect of BRC on P450s have been limited (Moochhala et al., 1989) with even fewer reports on the direct *in vitro* effect of BRC on CYP3A-catalyzing activity.

In the adult male rat, CYP3A2 is constitutively expressed and makes up a large portion of P450 enzymes in the liver. In addition to its prevalent expression in the liver, CYP3A2 shares a 72% amino acid homology with the human isoform CYP3A4 (Maurel, 1996), which catalyzes the breakdown of several key drugs including: acetaminophen, cyclosporine, erythromycin, warfarin, theophylline, and tamoxifen (Parkinson, 2001). One of the markers for catalytic activity for this isoform is the formation of 6β-hydroxytestosterone (6β-OHT) from testosterone. The metabolites, 2α-OHT and 16α-OHT, are products of CYP2C11-catalyzed metabolism of testosterone.

The present study investigated the inhibitory characteristics of BRC on CYP-catalyzed reactions with testosterone. We show that BRC is a competitive and selective inhibitor of CYP3A-catalyzed testosterone metabolite formation.

A1.2 Materials and Methods

A1.2.1 Chemicals

All reagents used in the HPLC assay including methanol, acetonitrile, and dichloromethane were purchased from EM Science (Gibbstown, NJ, USA) in the highest purity available. BRC was purchased in the form of 2-bromo-α-ergocryptine methanesulfonate salt (Sigma-Aldrich, St. Louis, MO, USA) and dissolved in methanol. Testosterone (4-Androsten-17β-ol-3-one), 11α-hydroxyprogesterone, magnesium chloride, potassium phophate, β-nicotinamide adenine dinucleotide (NADP), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Aldrich (St. Louis, MO, USA) in the highest purities available. Testosterone, 11α-hydroxyprogesterone, and testosterone metabolites were all dissolved in HPLC-grade methanol. All testosterone metabolites were also purchased from Sigma Aldrich, with the exception of 16α-hydroxytestosterone, which was purchased from Steraloids (Newport, RI, USA).

A1.2.2 Microsome isolation

Upon sacrifice of animals, the liver was immediately excised. Liver microsomal isolation was achieved with the use of a previously described method of differential centrifugation (Coon et al., 1978a) and kept at 4°C during the entire preparation. Liver tissue was ground in three volumes of Tris chloride buffer

consisting of 0.1 M EDTA and 0.15 M potassium chloride using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA, USA). Samples were then centrifuged at 9,000 x *g* for 20 minutes at 4°C. The supernatant was collected and centrifuged at 550,000 x *g* for 17 minutes at 4°C. The supernatant was discarded and the pellet resuspended in sodium pyrophosphate buffer containing 0.1 M EDTA at pH 7.4. The suspension was then homogenized and afterward centrifuged at 550,000 x *g* for another 17 minutes at 4 ºC. The supernatant was discarded and the pellet washed and homogenized in Tris buffer containing 20% glycerol for storage. The microsomes were then stored at –80°C until analysis.

A1.2.3 Testosterone Hydroxylation Assay

The testosterone hydroxylation assay using liver microsomal samples was performed as previously described (Brunner et al., 1996b) and is a modification on a prior system that had been developed (Sonderfan et al., 1987). In brief, 200 μg of liver microsomal protein was included in a 1 ml reaction mixture containing 0.02M potassium phophate buffer (pH 7.4), water, and an NADPH regeneration system consisting of 0.5 mM β-nicotinamide adenine dinucleotide (NADP), 10 mM glucose-6-phosphate, 10 mM magnesium chloride, and 5 units glucose-6-phosphate dehydrogenase. The substrate, testosterone, was added at the following concentrations: 50, 100, 200, and 400 μM. Within 5 seconds of adding the substrate, the inhibitor, bromocriptine was added at the following concentrations: 50, 5, 0.5,

0.05, 0 μM. After a 3-minute pre-incubation with both testosterone and bromocriptine, the samples were then incubated with glucose-6-phosphate dehydrogenase for 15 minutes under the same conditions to initiate the reaction. The reaction was quenched with 5 ml of dichloromethane and 3.6 mmol of 11α-hydroxyprogesterone was added as the internal standard. Once the organic phase was transferred and evaporated, they were dissolved in 200 μl methanol and stored at 4^{o} C for no longer than 1 week before use.

A1.2.4 Chromatography

Separation and detection of testosterone and metabolites were performed on a Shimadzu HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) equipped with an automatic injection system (SIL-10A), dual solvent delivery pumps (LC-10AS), a system controller (SCL-10A), and a variable wavelength UV-VIS detector (SPD-10A). The system was operated by the Class-VP version 7.2 software package (Shimadzu Scientific Instruments, Columbia, MD, USA). Testosterone metabolites were resolved on a 150 X 4.6 mm C-18 column (Supelco, Belafonte, PA, USA) that was kept at a constant temperature of 40^{o} C. The binary mobile phase system consisted of solvent A at a 39:60:1 ratio of methanol:water:acetonitrile and solvent B at a 80:18:2 ratio of methanol:water:acetonitrile. The two mobile phases were run on a gradient curve at a flow rate of 1.5 ml/ml delivered over 40 min. The analytes were monitored at a

wavelength of 238 nm. Peak areas of corresponding hydroxylation metabolites were measured and compared to peak areas of the internal standard within the same run. Rates were calculated under conditions that were linear with respect to protein and time.

A1.2.5 Data analysis

The mean velocities (of three animals) for all concentrations and metabolites were entered into a graphical program. All graphs and kinetic parameters were generated with the aid of the Grafit program version 5.0 (Erithacus Software Ltd., Horley, Surrey, U.K.).

A1.3 RESULTS

Bromocriptine was investigated as a potential inhibitor of P450-catalyzed testosterone metabolism based on previous observations (Lu et al, 2004, in press). P450-dependent steroid metabolism has long been recognized as a direct indicator of P450 catalytic activity. More specifically, the hydroxylation of testosterone to produce 6β-OHT is indicative of the activity of CYP3A2, and to a lesser extent, CYP3A1 (Waxman et al., 1988a). The productions of 16α- and 2α-OHT are representative of CYP2C11 activity (Waxman, 1984b).

The K_m, V_{max}, K_i, and IC_{50} values for the production of each metabolite are presented in Table A1.1. Bromocriptine at a concentration of 5 μM inhibited CYP3A1/2-catalyzed production of 6β-OHT with an apparent K_i of 3.9 μM. To assess whether the inhibition is enzyme specific, we also examined the inhibitory effect of bromocriptine on CYP2C11-dependent productions of 16α- and 2α-OHT. The inhibitory consequences of bromocriptine on 16α- and 2α-hydroxylase were not as great with K_i values of 46.8 and 47.0 μM, respectively.

IC_{50} values, the inhibitor concentration required to decrease enzyme activity by 50%, are presented in Table A1.1. The IC_{50} for the CYP3A1/2-dependent hydroxylation of testosterone at the 200 μM concentration is 0.94 μM, while it is much higher for both CYP2C11-dependent hydroxylation products, indicating a greater inhibitory effect on CYP3A1/2 activity. In addition, Lineweaver-Burk plots reveal a competitive inhibitory effect of bromocriptine on the production of 6β-OHT, but not for 16α- and 2α-OHT (Figures A1.2 and A1.3).

A1.4 DISCUSSION

The CYP3A subfamily has clinical importance due to its high expression in the liver and intestine, extensive range of substrates (reviewed in (Parkinson, 2001), and prominence as one of the subfamilies that metabolizes most drugs in humans (Wrighton and Stevens, 1992). As a consequence of its broad substrate specificity,

the potential for drug interactions is substantial. Although induction of CYP3A can have harmful effects on the efficacy of a drug, the most significant of relationships is the inhibition of CYP3A since this can lead to drug toxicity caused by decreased metabolism.

In the present study, potential inhibitory effects of bromocriptine were investigated. Since bromocriptine may be used in combination therapy, it is of chief importance to consider its consequences on drug metabolism as it may affect the metabolism and/or efficacy of other drugs being administered. There is evidence in literature that bromocriptine is metabolized by the CYP3A subfamily (Matsubayashi et al., 1997; Peyronneau et al., 1994a), although the involvement of other isoforms has not been excluded. Though reports on the effects of bromocriptine on P450s are limited, one study found that bromocriptine interfered with the metabolism of tacrolimus (Christians et al., 1996). Since tacrolimus is metabolized by CYP3A4 in humans, bromocriptine may be inhibiting the activity of the CYP3A subfamily, thereby decreasing tacrolimus metabolism.

Bromocriptine was more potent at inhibiting CYP3A-dependent metabolism of testosterone than CYP2C11 catalytic activity as evidenced by the lower IC_{50} value. Although the IC_{50} for 6β-hydroxylase activity is relatively low for this study, other compounds including clotrimazole have an established IC_{50} that is approximately 100-fold lower (Turan et al., 2001). Thus, although bromocriptine

inhibits CYP3A-catalyzed reactions, it is only moderately potent. Based on Lineweaver-Burk plots, it is a competitive inihibitor of CYP3A activity as well.

Although results from this study suggest that bromocriptine may interfere with the metabolism of other drugs that are substrates for the CYP3A subfamily, these data did not take into account *in vivo* conditions including distribution, protein binding, or accumulation of bromocriptine in hepatoyctes that would contribute to the overall drug interaction effect. Nevertheless, IC_{50} values and a low K_i for 6β-hydroxylase from this *in vitro* study indicates that bromocriptine is a selective inhibitor of CYP3A-dependent testosterone metabolism in rat hepatic microsomes.

Table A1.1 Kinetic parameters for the effect of bromocriptine on the hydroxylation of testosterone by P450 enzymes.

P450	Reaction	K_m (μM)	V_{max} (μM)	K_i (μM)	IC_{50} (μM)
CYP3A1/2	6β-hydroxylation	213.9 ± 34.2	1.10 ± 0.04	3.9	0.94
CYP2C11	2α-hydroxylation	57.5 ± 25.9	0.80 ± 0.04	46.8	2.05
CYP2C11	16α-hydroxylation	60.1 ± 20.7	0.70 ± 0.03	47.0	6.32

Table A1.1

Kinetic parameters for all metabolites at substrate concentration of 200 nM and inhibitor concentration of 5 μM. Data represent parameters calculated from the mean velocity values of 3 animals and ± S.E. of the K_m or V_{max} from non-linear fit curves.

Figure A1.1 Percent of control activity curves

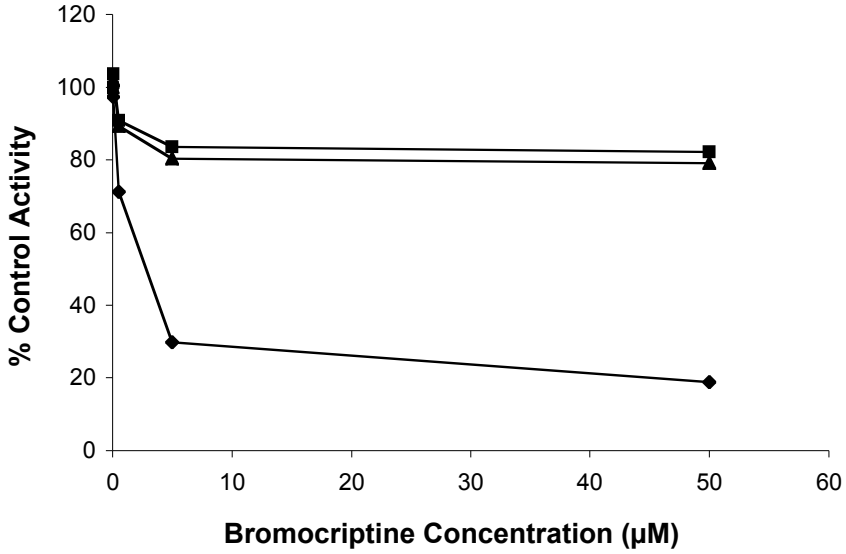


Figure A1.1 Percent of control activity curves for P450-catalyzed testosterone hydroxylation. Testosterone concentration was 50 nM. ♦ = 6β-OHT, ■ = 2α-OHT, ▲ = 16α-OHT. Each data point represents the mean from the data of 3 animals.

Figure A1.2 Bromocriptine selectively inhibits 6β-OHT production

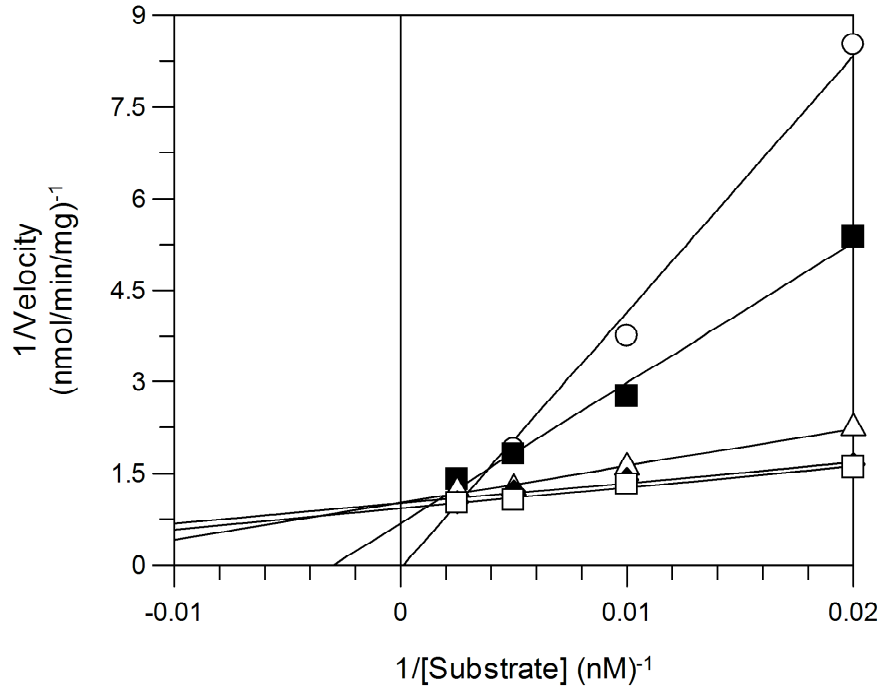


Figure A1.2

Lineweaver-Burk plot illustrating competitive inhibition of CYP3A-catalyzed testosterone hydroxylation by bromocriptine. Bromocriptine is represented at the following concentrations: ○ = 50 μM, ■ = 5 μM, △ = 0.5 μM, ◆ = 0.05 μM (obscured by □), □ = 0 μM. Each line represents the mean of n=3.

Figure A1.3 Bromocriptine's effects on 16α- and 2α-OHT production

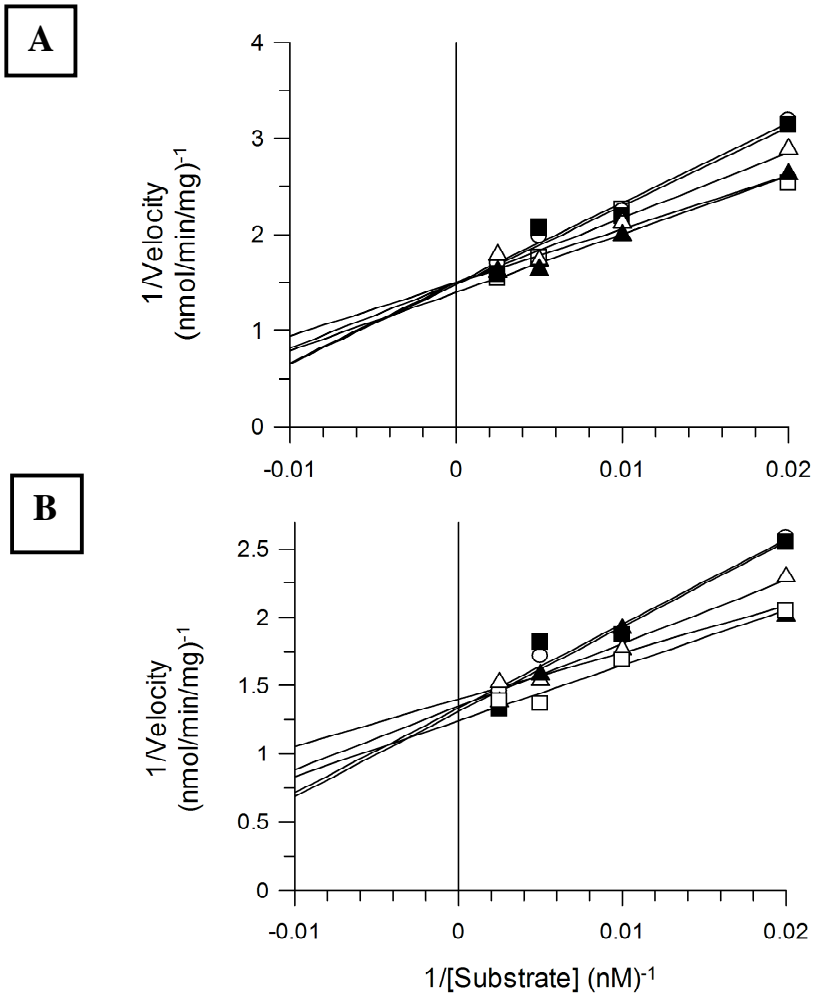


Figure A1.3

Lineweaver-Burk plots of the noncompetitive effect of bromocriptine co-incubation on the formation of 16α-OHT (A) and 2α-OHT (B). ○ = 50 μM, ■ = 5 μM, △ = 0.5 μM, ▲ = 0.05 μM, □ = 0 μM. Each line represents the mean of n=3.

APPENDIX II

Isolation of primary rat hepatocytes

I. Isolation procedure

A. Buffer and culture media preparation

1. Liver digest medium:

   Using 500 ml of liver digest medium (Invitrogen Life Technologies, Carlsbad, CA, USA), remove 25 ml of medium and add 25 ml of fetal bovine serum (FBS).

2. Liver perfusion medium:

   Using 500 ml of liver perfusion medium (Invitrogen Life Technologies, Carlsbad, CA, USA), remove 10 ml of medium and add 10 ml of penicillin/streptomycin (PCN).

3. Hepatocyte wash medium:

   From 500 ml of Williams' E medium (without L-glutamine), remove 12.55 ml of medium and add the following: 5 ml PCN, 5 ml L-glutamine, 2.5 ml of 1 M stock solution of HEPES, 55 μl of 1 mM stock of DEX in pure ethanol.

4. 90% isotonic percoll solution:

   Prepare a 10X solution of Dulbecco's PBS. Add 3 ml of 10X DPBS to 27 ml of Percoll (Amersham Biosciences, Piscataway, NJ, USA). Add 20 ml of PBS to this mixture to make final percoll solution.

5. Complete medium:

   From 500 ml of Williams' E medium (without L-glutamine), remove 42.55 ml of medium and add the following: 5 ml PCN, 5 ml L-glutamine, 25 ml of FBS, 2.5 ml of 1 M stock solution of HEPES, 55 μl of 1 mM stock of DEX in pure ethanol, 5 ml of ITS+ premix (BD Biosciences, Bedford, MA, USA).

6. Maintenance medium:

   From 500 ml of Williams' E medium (without L-glutamine), remove 10 ml of medium and add 5 ml PCN and 5 ml L-glut.

7. Growth factor reduced Matrigel preparation:

   All materials including pipettes and sterile tubes must be cooled to 4^{o} C prior to use to avoid solidification of Matrigel. Dilute Matrigel in cold Williams' E medium to make a concentration of 5 mg/ml. Add 400 μl of dilute Matrigel solution in dropwise fashion to each plate following the initial 3-h incubation of cells.

B. Surgical preparation and procedure

1. Prior to the start of the isolation, place 350 ml of the supplemented liver digest and liver perfusion media into separate sterile bottles in a 38º C water bath. Allow approximately 45 minutes for the solutions to warm up.
2. Once the buffers are warm, fill two 10-ml syringes with perfusion buffer in order to be used with the peristaltic pump (Harvard Apparatus, Holliston, MA, USA).
3. Place a rat weighing between 280 and 310 g into a restrainer and inject KAX (a cocktail of ketamine, acepromazine, and xyline) into muscle in the hindlimb.
4. Shave a large portion of the rat's abdomen as cleanly as possible and swab the entire area with alcohol pads to remove excess fur from the area.
5. Using autoclaved surgical instruments, carefully cut the skin, underlying fat layer, and muscle beginning at the genitalia and proceeding towards the heart in a U-shape. Avoid cutting any internal organs that may be attached to abdominal muscles.
6. Displace the intestines onto a stack of gauze at the same level of the body in order to maintain pressure on the hepatic portal vein.

7. Locate the hepatic portal vein on the caudal end of the liver and carefully isolate it using two sections of 4.0 suture thread approximately 6 cm in length each.
8. Secure the lower portion of the vein using hemostats attached to one of the pieces of suture tied into a loose knot.
9. Make a cut at a slight angle into the vein, being careful not to completely sever the vein.
10. Insert a cannula consisting of PE-50 and silastic tubing until the connector portion of the tubing is at the entry point at the initial cut.
11. Tie the tubing with the vein around it with the remaining piece of 4.0 suture thread. Make sure the cannula is connected to the tubing from the peristaltic pump.
12. Program the "push-pull" pump to deliver 350 ml of buffer at an initial rate of 18 ml/min.
13. After 1-2 minutes when the liver is swollen with buffer, make an incision in the superior vena cava at the level of the heart to allow for exit of the buffer.
14. Immediately following, increase the rate to 25 ml/min.
15. After blanching of the liver is observed at the faster flow rate, make an incision in the inferior vena cava at the level of the kidney in order to allow for additional exit of fluid.

16. Once the perfusate is clear upon exit, or approximately 300 ml of perfusion buffer has perfused, switch the pump to begin dispensing digest buffer.
17. After perfusion of both solutions is complete, perfuse the liver with perfusion medium until the perfusate is clear of phenol red.
18. Carefully excise the liver and place it into a sterile bottle containing 200 ml of Leibovitz L-15 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) on ice.

C. Digestion of liver and isolation of hepatocytes

1. Transport the liver to a laminar flow hood in a bottle containing Leibovitz medium on ice.
2. Remove the liver from the bottle and place it into a large sterile culture dish along with an excess of medium.
3. Using a sterile scalpel blade and forceps, mince the liver tissue into as fine of pieces as possible, making sure to keep the tissue from drying at all times by adding transport medium if necessary.
4. Using the circular flat end of a pestle, press the digested tissue through a sterile mesh screen attached to a sterile wide-mouth bottle.

5. After all of the cell suspension is collected in the bottle, divide suspension into four 50-ml centrifuge tubes and centrifuge them at 50 x *g* for 5 minutes at 4° C.
6. Resuspend the cells in cold wash buffer (supplemented).
7. Centrifuge the suspension under the same conditions as before.
8. After the resuspension spin, add 25 ml of 90% isotonic Percoll to two 50-ml centrifuge tubes and slowly add the hepatocytes from the last resuspension so as to create an undisturbed layer of cells above the percoll solution.
9. Centrifuge the percoll mixture at 1000 X g for 20-30 minutes at 4° C.
10. During this spin, place 10 ml of WEM onto each Matrigel plate and place in the incubator at 37° C and 5% CO_2.
11. Discard the supernatant and remove the brown, solid, middle layer and put it into fresh tubes.
12. Wash this pellet twice in hepatocyte wash medium as done previously. (Limit cells' exposure to Percoll as it is toxic.)
13. Resuspend the live cells in 25 ml of supplemented complete medium after last spin.
14. Aliquot 50 µl of suspension solution (1 from each tube) into correspondingly numbered bullet tubes.

15. From this set of tubes, number another set and aliquot 38 µl of PBS, 20 µl of sample (thoroughly mixed), and 2 µl of trypan blue into each tube. Do not add trypan blue until immediately before counting.
16. Pipette approximately 20 μl of the first sample into each chamber of the hemocytometer. Count all cells within 4 quadrants that have not incorporated trypan blue into the nucleus.
17. Calculate the concentration needed for 100% confluence plating (1.5 X 10^7 cells/plate) for 10 ml/per 100 mm plate. Typical cell yields range from 2 X 10^8 cells to 4 X 10^8 cells.
18. Remove Matrigel plates from the incubator and remove the WEM on the plates. Add 10 ml of cells to each dish.
19. Put plates back into incubator for 3-4 hours to allow for cell attachment.
20. Remove medium from dishes and replace with 9.6 ml of supplemented maintenance medium.
21. Add 400 μl of prepared 5 mg/ml growth factor reduced Matrigel matrix solution onto each plate in a dropwise fashion to form a Matrigel overlay.
22. Replace culture medium with maintenance medium (without Matrigel) every 24 hours following the first day. Figure A2.1 shows

freshly isolated hepatocytes on Matrigel plates 24 hours following plating.

II. Preparation of cellular extracts

A. Preparation of lysis buffer

1. Dissolve 0.788 g of Tris into 100 ml of purified water and adjust the pH to 7.4 with HCl.
2. Add the following components into the 50 mM Tris-HCl, pH 7.4 solution to make a total volume of 100 ml:
    a. 0.877 g NaCl (150 mM)
    b. 0.648 g β-glycerophosphate (30 mM)
    c. 0.074 g EDTA (2 mM)
    d. 0.019 g EGTA (0.5 mM)
    e. 1 ml NP-40 (1%)
    f. 0.5 g SDS (0.05%)
    g. 0.5 g sodium deoxycholate (0.5%)
3. Immediately before use, aliquot 8.5 ml of this stock solution and add the following components to make a total of 10 ml:
    a. 100 μl of 100 mM stock of DTT (1 mM)
    b. 100 μl of 100 mM stock of PMSF in DMSO (1 mM)
    c. 50 μl of 200 mM stock of Na_2VO_4 (1 mM)

d. 1 ml of 0.5 M stock of NaF (50 mM)

e. 20 μl of 5 mg/ml stock of pepstatin A

f. 10 μl of 10 mg/ml stock of soy trypsin inhibitor (10 μg/ml)

g. 50 μl of 5 μg/ml stock of aprotinin (5 μg/ml)

h. 100 μl of 100 mg/ml stock of Pefabloc SC (1 mg/ml)

i. 100 μl of 10 mg/ml stock of leupeptin (10 μg/ml)

4. Place the working lysis buffer on ice.

B. Removal of cells from plates and cell lysis

1. Prior to starting, place one bottle of sterile PBS on ice for several hours. Place another bottle of sterile PBS at room temperature.
2. Remove plates from the incubator and remove all media from plates.
3. Rinse plates with approximately 10 ml of room temperature PBS and remove. Repeat rinse.
4. Place approximately 10 ml of ice-cold PBS onto each plate.
5. Using a plastic cell scraper, scrape cells starting from the edge in a circular pattern while scraping into the circle with each revolution until all cells are removed.
6. Transfer PBS liquid containing matrigel and cells into a sterile centrifuge tube. Repeat with ice-cold PBS.

7. Place all tubes into centrifuge and spin for 5 mins. at 2,000 x *g* and 4° C.
8. Discard the supernatant and add between 175-250 μl of working lysis buffer into each tube.
9. Transfer pellet and lysis buffer into a microcentrifuge tube.
10. Using a 1 ml syringe with a 21 gauge needle attached, withdraw and release the cells through the needle repeatedly for a total of 25 times.
11. Place cells on ice for 40 mins.
12. Centrifuge the cells at 14,000 x *g* for 20 mins. at 4° C.
13. Transfer the supernatant from this spin containing cellular extracts into pre-labeled tubes and store at -80° C until use.

Figure A2.1 Primary rat hepatocytes

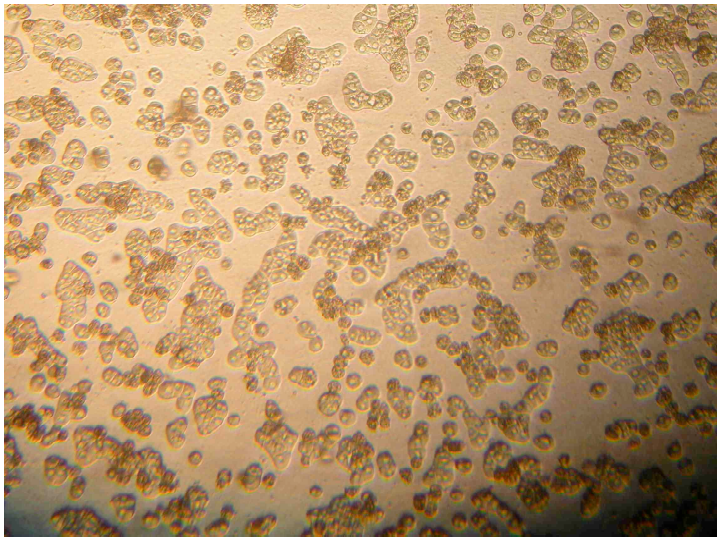


Figure A2.1

Primary rat hepatocytes twenty-four hours post-plating on thin-layer Matrigel substratum with growth-factor reduced Matrigel overlay. The medium consisted of Williams' E medium supplemented with only L-glutamine and penicillin/streptomycin.

APPENDIX III

Methods validation

A. Western blotting

Western blotting techniques were validated with respect to protein content and relative density values. The isoforms tested were CYP3A1/2 and CYP2C11. Gels were prepared and loaded as described in chapter 2. Three different samples were loaded in triplicate at the following protein amounts: 0, 10, 20, 40, and 80 μg. All samples were analyzed using a microsomal protein sample derived from the livers of either a phenobarbital-treated rat (for induction of CYP3A2 expression) or a dexamethasone-treated rat (for induction of CYP2C11 expression) as a protein standard. The density of each sample was divided by the mean of two standard densities from the same gel. The linearity for CYP3A1/2 is presented in Figure A3.1 and in Figure A3.2 for CYP2C11.

B. *In vitro* testosterone hydroxylation assay

Testosterone hydroxylations were carried out as previously described in chapter 2. Validations were conducted with respect to protein content vs. rate of

metabolite production and with respect to intraday and interday variability. The productions of 6β-hydroxytestosterone (6β-OHT), a marker for CYP3A1/2 catalyzed activity, and 2α-hydroxytestosterone (2α-OHT), a marker for CYP2C11 catalyzed activity were measured. To determine protein content linearity, rates of metabolite formation corresponding to the following protein amounts were assayed in triplicate: 1000, 500, 250, 125, 62.5, and 31.3 mg. The range of linearity where the correlation, r^2, was greater than 0.999, was determined to be between 31.3 and 500 mg for 6β-OHT production (Figure A3.3) and between 125 and 500 mg of protein for 2α-OHT (Figure A3.4). The limit of quantitation of this HPLC assay was 0.15 μg/ml, while the limit of detection (at a signal to baseline noise ratio of 3:1) was 0.05 μg/ml.

Intraday variability was tested by using two of each previously determined low, medium, and high activity-containing protein samples. A group of six samples (2 low, 2 medium, and 2 high) was assayed and repeated in quadruplicate within the same day (Table A3.1). All rates of metabolite formation are presented in nanomoles per minute per milligram of total microsomal protein. Interday variability was tested on four consecutive days and included one representative sample from each classification, low, medium, and high, assayed in duplicate.

Figure A3.1 Protein content linearity for CYP3A1/2 Western blot

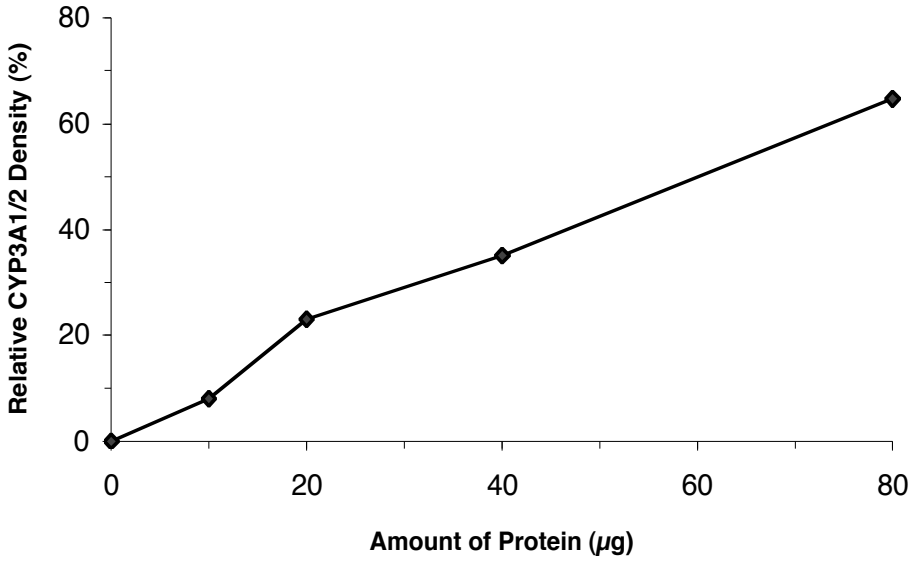


Figure A3.1

Hepatic CYP3A1/2 Western blot linearity with respect to amount of protein and relative density percentage. Relative density percentage is calculated as the raw density of each sample divided by the density of the standard and expressed as a percentage.

Figure A3.2 Protein content linearity for CYP2C11 Western blot

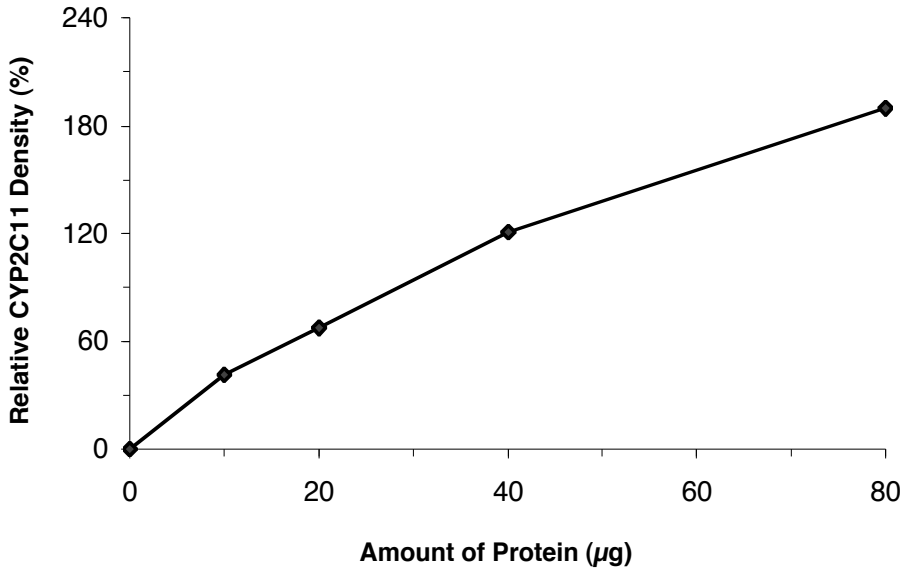


Figure A3.2

Hepatic CYP2C11 Western blot linearity with respect to amount of protein and relative density percentage. Relative density percentage is calculated as the raw density of each sample divided by the density of the standard and expressed as a percentage.

Figure A3.3 Protein content linearity for the production of 6β-OHT

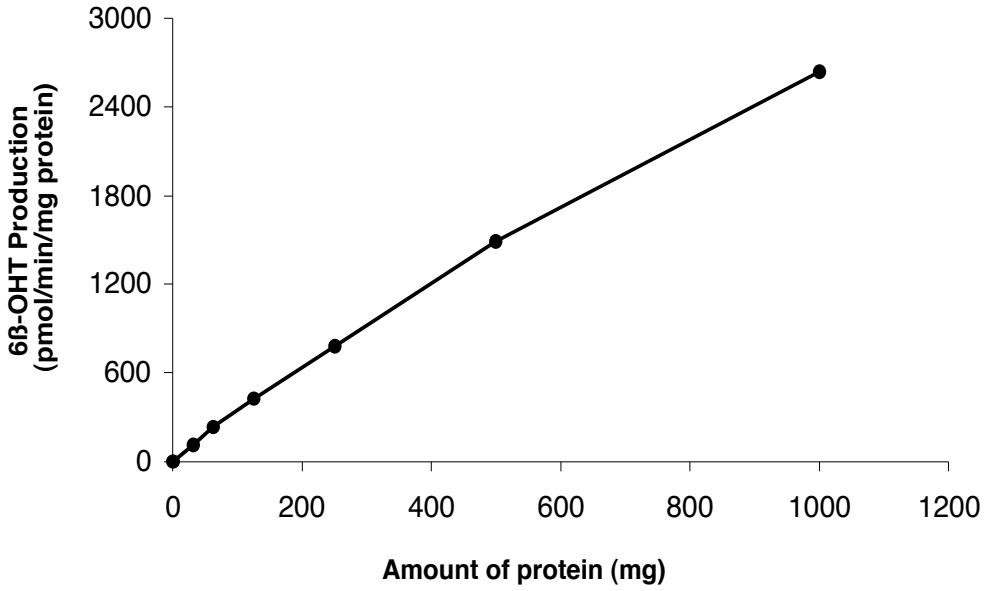


Figure A3.3

Protein content linearity with respect to protein content and 6β-OHT production using a testosterone hydroxylation assay followed by HPLC analysis. Range of linearity where $r^2 > 0.999$ is between 31.3 and 500 mg of protein.

Figure A3.4 Protein content linearity for the production of 2α-OHT

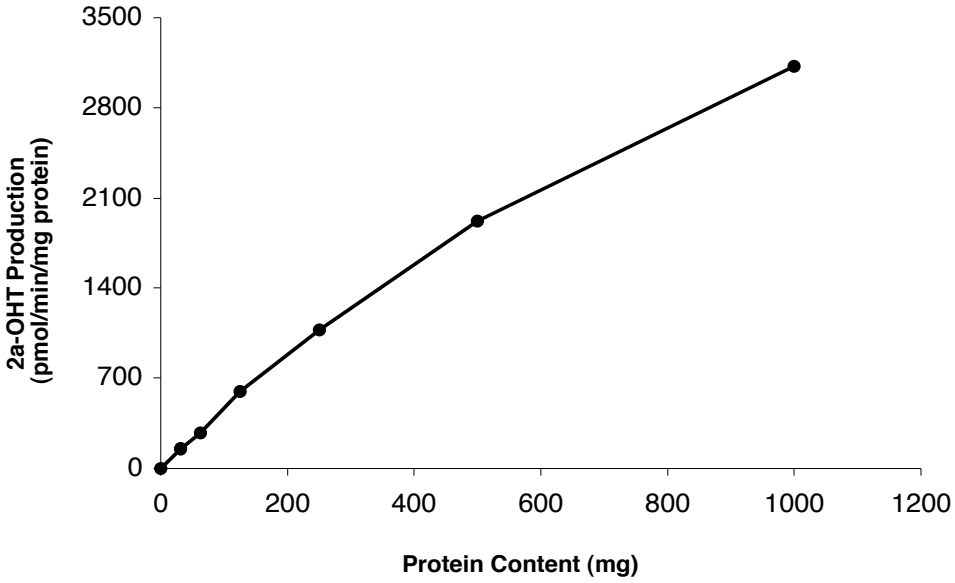


Figure A3.4

Protein content linearity with respect to 2α-OHT production using a testosterone hydroxylation assay followed by HPLC analysis. Range of linearity where $r^2 > 0.999$ is between 125 and 500 mg of protein.

Table A3.1 Intraday and interday variabilities in the productions of 6β-OHT, 2α-OHT, and 7α-OHT

Intraday Variability	Range	Mean ± SD	%CV
6β-hydroxy-testosterone	Low	40.30 ± 4.63	11.50
	Medium	529.01 ± 6.09	1.15
	High	1311.42 ± 7.44	0.57
2α-hydroxy-testosterone	Low	51.70 ± 6.43	12.44
	Medium	632.04 ± 13.58	2.15
	High	1100.48 ± 13.91	1.26
7α-hydroxy-testosterone	Low	32.93 ± 3.96	12.02
	Medium	88.42 ± 3.65	4.13
	High	93.41 ± 2.79	2.99

Interday Variability	Range	Mean ± SD	%CV
6β-hydroxy-testosterone	Low	47.41 ± 1.92	4.04
	Medium	545.52 ± 13.64	2.50
	High	1363.01 ± 33.01	2.42
2α-hydroxy-testosterone	Low	52.34 ± 2.80	5.35
	Medium	653.14 ± 10.31	1.58
	High	1122.61 ± 22.75	2.03
7α-hydroxy-testosterone	Low	29.88 ± 3.41	11.40
	Medium	75.22 ± 5.88	7.80
	High	81.04 ± 5.86	7.20

ABBREVIATIONS

ANOVA – analysis of variance

AP-1 – activator protein-1

AUC – area under the curve

BRC – bromocriptine

cAMP – cyclic adenosine monophosphate

Clcr – creatinine clearance

CsA – cyclosporine

DTT – dithiothreitol

EDTA – ethylenediamine tetraacetic acid

EGTA – ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

FBS – fetal bovine serum

GH – growth hormone

GHR – growth hormone receptor

GHRH – growth hormone releasing hormone

GHRP – growth hormone-releasing peptides

HEPES – 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

HPLC – high performance liquid chromatography

IGF-I – insulin growth factor-1

IL – interleukin

JAK2 – Janus kinase 2

JNK – c-Jun NH2-terminal kinase

MSG – monosodium glutamate

MTS – (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

NF-AT – nuclear factor of activated T cells

NFDM – non-fat dry milk

NF-κB – nuclear factor-Kappa B

NP-40 – Nonidet® P40 substitute

P450 – cytochrome P450

PBS – phosphate-buffered saline

PMSF – phenylmethylsulfonyl fluoride

PRL – prolactin

SDS-PAGE – sodium docecyl sulfate-polyacrylamide gel electrophoresis

SRIF – somatotropin release-inhibiting factor

Stat5b – signal transducers and activators of transcription 5b

TBS – Tris-buffered saline

WEM – Williams' E medium

X-OHT – X designates the regio- and stereochemistry of the testosterone hydroxylation metabolite (*e.g.*, 6β-OHT denotes 6β-hydroxytestosterone)

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-**Lu, S.K.,** Croyle, M.A., Callahan, S.M., and Brunner, L.J.: Up-regulation of the JAK/Stat pathway by cyclosporine in primary rat hepatocytes. Manuscript in preparation.

-Lu, S.K., Callahan, S.M., and Brunner, L.J.: Bromocriptine is a competitive and selective inhibitor of CYP3A-catalyzed hydroxylation of testosterone. Manuscript in submission, *Drug Metabolism and Disposition*.

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